

**NUTRITIONAL TOXICITY OF SULFUR AND  
SULFUR-NUTRIENT INTERACTIONS IN RUMINANTS**

**A DISSERTATION**

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by

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## ABSTRACT

High dietary sulfur (S) in ruminants introduces a range of effects not seen in non-ruminant animals. This thesis focuses on several new mechanisms through which S interacts with copper (Cu) and thiamine metabolism, and on more complex three way interactions of S-Cu-thiamine in ruminants. The present investigation comprises three field studies, one in vivo study, and one in vitro study.

The field studies were conducted on Saskatchewan farms. These studies include investigations of: a) the effects of high sulfate in the drinking water on blood thiamine status, and b) nutritional aspects and selected biochemical parameters in herds with incidence of clinical problems associated with consumption of excess S in drinking water. Cattle exposed to high levels of S in drinking water had lower blood thiamine than those drinking water containing a low concentration of S. The occurrence of polioencephalomalacia (PEM) was documented in all of our field research. These studies also showed that Cu influences interactions between S and thiamine. Both Cu and thiamine depletion in the blood of cattle drinking water containing a high concentration of S can be corrected by dietary supplementation of Cu alone.

Chronic effects of nutritional S toxicity were investigated using sheep as a model. This study confirmed the known effects of S on Cu metabolism. It was also observed that Cu influences the effect of S on thiamine metabolism, and that the interaction of S-Cu-thiamine occurs in the gastrointestinal tract as well as systemically. The in vivo study also showed that, in chronic nutritional S toxicity

in ruminants, the target organ is the brain. The early, (subclinical) morphologic and metabolic lesions were evident as altered electrophysiological events along the auditory pathway. After prolonged exposure (three to eight weeks) several animals developed PEM. The incidence of PEM was attributed ( $p < 0.05$ ) to excess dietary S. There were no changes in transketolase (TK) activity in the tissue and only mild to moderate changes in the blood TK in animals fed excess S. However, dietary thiamine supplementation in sheep fed high S diets prevented manifestation of clinical signs and gross brain lesions, but did not prevent microscopic lesions. Thus, the S-induced PEM does not appear to be an effect of thiamine deficiency per se. Changes in several biochemical parameters observed in high S-related PEM in sheep and cattle appear to be similar to those observed in the other "spontaneously" occurring field cases and amprolium-induced PEM in ruminants. No pathognomonic diagnostic markers, among commonly used blood chemistry indices, enabling early recognition of the problem were found. Interestingly, excess dietary S affects microbicidal activity of neutrophils, and the S-related impairment of the neutrophil function is to be attributed to an apparent subclinical thiamine deficiency.

The in vitro study was conducted to investigate the effects of S on thiamine metabolism and thiamine destroying activity in the rumen. This study showed that excess S has a destructive effect on thiamine in rumen cultures in vitro, potentially through a thermolabile factor. The effect of S on the overall thiamine status may be modest when the content of thiamine in the feed is high or when

the ruminal synthesis is extensive. However, should any of the above factors be inadequate, the S may have a physiologically significant effect. This is consistent with the observations in vivo.

It can be concluded from these studies that:

- 1) Animals exposed to excess dietary S appear to have an increased metabolic requirement for thiamine, and therefore thiamine deficit is one of the key features associated with nutritional S toxicity problems.
- 2) Copper status likely plays an important role in the metabolism of thiamine.
- 3) Thiamine status indicative of deficiency is not necessarily the cause of PEM, although thiamine inadequacy may increase the risk of developing the clinical signs of PEM.
- 4) Sulfur-induced PEM is not likely caused by overt thiamine deficiency based on commonly used criteria. The morphological damage is not likely due to the metabolic effects of decreased activities of TPP-dependent enzymes. Thiamine may play some (unknown) protective role apart from its metabolic effect through thiamine dependent enzymes, since elevated thiamine status prevents development of clinical signs of PEM in animals fed excess S.
- 5) Evidence was obtained that S affects the bioavailability of thiamine in the rumen. Some indirect evidence of possible systemic effects from a factor interfering with thiamine metabolism was observed in the present study.
- 6) Animals exposed to excess dietary S would benefit from thiamine supplementation.

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## LIST OF ABBREVIATIONS

Ca - calcium

Fe - iron

Mg - magnesium

P - phosphorus

Zn - zinc

TM - thiomolybdates

B<sub>1</sub> - thiamine

CPK - creatinine phosphokinase

GGT - gamma-glutamyltransferase

PEM - polioencephalomalacia

CNS - central nervous system

BAER - brain stem auditory evoked response

L - liter

μL - microliter

g - gram

μg - microgram

h - hour

DM - dry matter

GI - gastrointestinal

Cl - chlorine

K - potassium

Na - sodium

S - sulfur

Mo - molybdenum

Cu - copper

TPP - thiamine pyrophosphate

AST - aspartate aminotransferase

TK - transketolase

CCN - cerebrocortical necrosis

mL - milliliter

kg - kilogram

mg - milligram

nm - nanometre

min - minute

## INTRODUCTION

### **Background: Practical significance of nutritional toxicity of sulfur**

The sulfate content of water on many farms throughout the Canadian Prairies is considered to be excessive for animals. Thus, large numbers of animals raised on the prairies are exposed to potentially toxic levels of sulfur (S) in drinking water. In view of this, the problem of S toxicity may be important for the livestock industry. A better understanding of the mechanisms of the toxic effects resulting from excessive S intake would be valuable, particularly from an economical and clinical management standpoint.

The detrimental effects of high dietary S are of particular importance in ruminants. The biochemical and biological conditions in the rumen provide an ideal environment for numerous interactive processes which can often lead to clinical manifestation of metabolic disorders. High S intake interferes with copper (Cu) metabolism and has been reported to be involved in the induction of polioencephalomalacia (PEM). An epidemiological study conducted by Raisbeck (1982) indicated a very strong positive relationship between occurrence of PEM and feeding excess dietary S. According to this study PEM is 43 times more likely to be diagnosed in a herd fed high S rations than in herd not fed high S ration. Polioencephalomalacia is one of the most common metabolic neurodegenerative disorders of ruminants affecting the central nervous system (CNS). Morphologically, PEM is characterized by a focal necrosis of the grey

matter localised in various structures of the brain. The lesions are qualitatively similar in all cases of PEM, but it is not clear whether or not PEM has any specific common etiology. However, there is an ample body of evidence that thiamine insufficiency or disturbances in thiamine metabolism are implicated in the pathogenesis of this syndrome. Thiamine, if administered in early stages of the disease, leads to prompt and complete clinical recovery. It is commonly believed that the disease is due to thiamine deficiency.

The above facts led us to formulate a hypothesis that high dietary S affects thiamine status in ruminants. The hypothesis was tested in a field study following the incidental development of PEM in feedlot cattle drinking water containing a high level of S. The results showed that many animals had a very low concentrations of thiamine and Cu in blood. Supplementation of Cu to these animals markedly improved both Cu and thiamine status. This observation indicated possible interactions among Cu, S, and thiamine. Following this, the full investigation using further field studies, in vivo experiments, and in vitro experiments was undertaken to determine the metabolic effects of excess dietary S and basic mechanisms of Cu, S and thiamine interactions.

## **Objectives**

### **General:**

- 1) To investigate the effects of excess dietary sulfur on copper and thiamine metabolism, several selected biochemical parameters, and overt toxicity in ruminants.

2) To study the effects of S on thiamine metabolism in the rumen.

**Specific:**

1) To study the effects of S on thiamine and Cu status, and selected biochemical parameters in cattle where herd health problems were associated with dietary S toxicity.

2) To study the effects of dietary S under controlled experimental conditions on:

a) thiamine and Cu metabolism and metabolic interactions among S, Cu, and thiamine.

b) indices of thiamine and Cu status in blood and tissues

c) selected biochemical parameters

d) electrophysiological events in the brain

e) selected immune functions of polymorphonuclear neutrophils

3) To study interactions of S and thiamine in the rumen with emphasis on:

a) effects of S on metabolism of thiamine in the rumen

b) thiamine-destroying activity of S in the rumen

## GENERAL HYPOTHESIS

There is a complex array of interactions among S, Cu, and thiamine. These nutrients interact both in the gastrointestinal tract and systemically. In severe cases, the target organ of S toxicity is the brain. The effect of S on brain metabolism is a complex chain of metabolic events which are triggered by the formation of toxic S metabolites originating in the rumen and the alteration of thiamine metabolism. Sulfur in the rumen affects the thiamine molecule, altering it and rendering it biologically inactive and capable of competing with the intact (active) thiamine molecule. A state of metabolic thiamine insufficiency can occur under these circumstances. This may result in an impairment of metabolic events which depend on thiamine. Excess dietary S may lead the production of S-derived metabolites which may cause damage to the brain tissue. Toxic effects of S-derived metabolites and some form of metabolic thiamine inadequacy may be responsible for development of malacic lesions and clinical signs.

## LITERATURE REVIEW

### 1.1 Sulfur (S)

#### 1.1.1 Chemistry of S

Sulfur is the element in group VIA of the periodic system. Elemental S is a tasteless, yellow solid at physiological temperature and pressure. It is one of the more abundant elements present in the earth, oceans, and the atmosphere. Sulfur can be found in a variety of chemical forms. The electronic configuration allows sulfur to assume a number of valencies at a number of oxidation states, ranging from -2 to +6. More often occurring in nature are: elemental S, and a plethora of organic and inorganic forms in oxidation states as sulfides, sulfites, and sulfates. Biological compounds of S are diverse. Sulfur enters the biosphere oxidized to sulfate. The reduction of inorganic sulfate to sulfide and the subsequent oxidation of sulfide to sulfate forms the biological sulfur cycle.

##### 1.1.1.1 Analytical methods

The diversity of S compounds requires a highly specific method of analysis. A variety of methods have been described. Total sulfur can be analyzed by induction coupled plasma emission spectroscopy (Blanchar et al. 1965). Sulfate analysis can be performed using turbidimetric or nephelometric methods involving the production of a suspension of barium sulfate as described by Sörbo (1987). The chromatographic method for sulfate measurements has also been described (Reiter et al. 1987). A liquid chromatographic method can be used for determination of sulfite (Kim 1990, Lawrence et al. 1990). Balasubarian and

Kumar (1990) presented a very sensitive spectrophotometric method for the determination of trace amounts of hydrogen sulphide after fixing in a modified zinc acetate-disodium ethylenediaminetetraacetate-sodium hydroxide solution. Alternative methods for determination of hydrogen sulfide and total sulfide have been described by Florin (1991). Sulfide can also be measured by precolumn derivatization with N,N-dimethyl-paraphenyldiamine to produce methylene blue and reverse-phase high pressure liquid chromatography as described by Savage and Gould (1990).

### **1.1.2 Biology of S**

#### **1.1.2.1 Sulfur as a required element**

In the process of evolution S became indispensable to sustain life at the very early stages of development of living matter. It has been suggested that elemental S vapors could protect the biosphere against solar UV radiation in an anoxic, ozone-free, primitive atmosphere in a similar fashion as the present biosphere is shielded by atmospheric ozone (Kasting et al. 1989). Presumably, energy metabolism based on S may have preceded metabolism based on oxygen. Dissimilatory sulfate reduction was occurring some 3.5 billion years ago. This was soon after the emergence of life on earth (about 4 billion years ago). Free oxygen appeared and accumulated in the atmosphere about 1 to 2 billion years ago, that is after the development of the photosynthetic activities of green plants (Roy and Trudinger 1970). A warm, sulfur-rich, primitive atmosphere is consistent with inferences drawn from molecular phylogeny, which suggest that

some of the earliest organisms were thermophilic bacteria that metabolized elemental sulfur (Kasting et al. 1989). Some contemporary strains of bacteria obtain energy for growth by the oxidation of organic molecules or hydrogen and reduction of sulfate to sulfide (Badziong and Thauer 1980).

#### **1.1.2.2 Biological importance of S**

The above and following information, if not specifically referenced in the text, was obtained from Roy and Trudinger (1970) and Huxtable (1986). In all forms of life, S is a building element of numerous biologically active compounds. All living organisms are susceptible to S deficiency and a prolonged state of S deficiency, if not corrected, will inevitably lead to death of the organism.

The essential role of S is largely determined by its biochemistry. The thiol (-SH) group is among the most reactive groups occurring in the living cell. The enzymatic reduction (-SH) or oxidation (-S-S-) of thiols provides an extremely versatile arrangement which is of central importance to cellular structure and function. The disulfide bond (-S-S-) is one of the key chemical structures of living matter. In proteins, this bond is crucial for activity and structural integrity. Thiols are also involved in other biological functions such as storage, transport, detoxification and protection of the cell against toxic metabolites. The ability of thiols to form stable complexes with metals is of biochemical, therapeutical and toxicological importance. Cysteine is a component of biologically active molecules where oxidation or reduction of -SH groups is a key reaction. From a chemical perspective, this amino acid has enormous versatility due to the presence of three

ionizable groups which are affected by the specific environment. The ready oxidizability of thiols defines their biological functioning. Biologically active compounds such as flavins, cytochromes, dehydroascorbate etc., can accept electrons from a thiol, oxidizing it into a disulfide. The other S-containing amino acid is methionine. It is utilized mainly as a building block in protein synthesis. Methionine, via its metabolite S-adenosylmethionine, also serves as a methyl group donor for numerous transmethylation reactions.

Thiol groups, because of their nucleophilicity, are among most reactive intracellular sites performing an important function in detoxifying processes. Exposure to alkylating agents results in their complexing with low-molecular weight thiols, of which glutathione is most abundant. Glutathione is present at high concentrations in cells of all living organisms. It protects the cell from toxins of endogenous and xenobiotic origin.

Sulfur is a building element of numerous biological molecules required in metabolic reactions. Coenzyme A is the major thioester taking part in all the major pathways for producing energy as well as the synthesis of structural components of the cell. Lipoic acid occurs in multienzyme complexes responsible for oxidative decarboxylation of ketoacids. In its biochemical function, lipoate oscillates between the oxidized and the reduced form of the disulfide ring. Thiamine is a complex molecule composed of pyrimidine and S containing thiazole moieties. This vitamin is involved in nonredox enzymatic reactions being of importance in carbohydrate energy metabolism. Biotin is another thioether

vitamin. Biotin-dependent enzymes are involved in carboxylation, transcarboxylation and decarboxylation reactions. The major pathways are involved in the synthesis of fatty acids and in gluconeogenesis.

One of the oxidation states of S most represented in nature is sulfate. Sulfates of organic molecules occur in enormous variety and abundance in living organisms. Sulfated lipids and polysaccharides are incorporated in the structure of the cellular wall and connective tissue. Most represented are chondroitin sulfate, heparin, proteoglycans and cerebroside. Sulfate moieties of sulfated biomolecules increase their water solubility and facilitate transport and storage, are involved in water retention, electrolyte transport, increase structural rigidity, facilitate macromolecular interactions and cell-to-cell interactions, and are involved in the creation of recognition sites. Sulfonation is also involved in the metabolism of xenobiotics by inactivating toxic elements and increasing their water solubility.

## **2.1 Sulfur metabolism in ruminants**

### **2.1.1 General consideration**

The metabolism of sulfur in ruminants is unique among mammals. The ruminant host combines a complex microbial system in the rumen with a mammalian metabolic system. Rumen microorganisms can reduce oxidized forms of S to forms which can be incorporated into organic compounds. Hence, ruminants have the ability to obtain their S supply from both organic and inorganic sources, benefitting from rumen microorganisms capable of reducing

S compounds to sulfide (Lewis 1954) and incorporating inorganic S into cellular materials (Anderson 1956, Hendericks 1961). Non-ruminant animals must obtain their dietary supply of S in an organic form.

#### **2.1.1.1 Bioavailability of S**

The biological availability of S from L-methionine (Johnson et al. 1971) and DL-methionine (Albert et al. 1956, Kahlon et al. 1973) is higher than S from other sources. Orally administered cysteine and methionine are almost completely degraded in the rumen (Garrigus 1970), therefore they do not serve primarily as an amino acid source to the animal, but as a source of S. Also analogues of methionine have high biological availability as a source of S (Bouchard and Conrad 1973a, Bull and Vandersall 1973, Kahlon et al. 1973). Several sources of inorganic S have proven to be available to the ruminant, but in general the S from inorganic sources is regarded as less available than from methionine. The group of sulfates with relatively high S availability includes sodium sulfate, calcium sulfate, potassium and magnesium sulfate, and ammonium sulfate. The availability of S from sodium sulfate is 80% of that from methionine (Johnson et al. 1971). Other reports showed that sodium sulfate is a good source of S (Bouchard and Conrad 1973bc, Bull and Vandersall 1973, Kahlon et al. 1973). Calcium sulfate has been reported to be equal to sodium sulfate as a source of S (Bouchard and Conrad 1973b, Kahlon et al. 1973). Potassium and magnesium sulfates were compared to sodium sulfate by Bouchard and Conrad (1973a) and were found to result in similar availability values for S. Sulfur in elemental S is

less available than that from most other sources. Elemental S has been shown to be about half as "available" as is sulfate S, whereas the sulfate S in water is probably nearly 100% available in the rumen (Bull 1985). Albert et al. (1956) concluded that over three times as much supplemental S was needed in the form of elemental S than an equivalent from methionine. Others confirmed lower S availability from elemental S (Goodrich and Tillman 1966, Kahlon et al. 1973). However, Chalupa et al. (1973) found elemental S equal to sulfate sulfur for cattle. The reason for such inconsistency of findings is unknown.

#### **2.1.1.2 Sulfur in cation-anion balance**

Dietary cation-anion balance, defined as the balance of positively and negatively charged ions in the diet, affects systemic acid-base status. In ruminant research, the influence of S on cation-anion balance has not been studied extensively, and thus far the research has been mainly focused on dairy cattle. However, the contribution of dietary S to overall dietary ions and its influence on acid-base balance should not be ignored. This problem definitely deserves more consideration.

In the past investigations S was omitted from the equation by some (Fredeen et al. 1988, Tucker et al. 1988), whereas other workers included it (Beighle et al. 1988, Block 1984). Recently, however, Thacker et al. (1991) suggested that dietary S may have a similar acid generating potential as chlorine. The excess dietary S may affect performance of animals. Daily milk yield and milk fat and protein content were decreased by dietary S supplementation (Tucker et al. 1991). The

latter authors suggest that it may be necessary to include a modifying coefficient for S in the cation-anion equation to adjust for S in an acid-generating potential, which can be dependent on the dietary source of S and a number of other dietary factors. The physiological importance of dietary S, as a generator of a strong anion, was also emphasised by Oetzel (1991). On the basis of the compiled data from 75 trials, the latter author concluded that dietary S is important in the cation-anion equation. According to Oetzel (1991) moderately elevated dietary S levels were associated with a lower incidence of milk fever. The reason for this remains obscure.

## **2.1.2 Sulfur metabolism in the gastrointestinal tract**

### **2.1.2.1 In the rumen**

Sulfur in either its inorganic or organic forms is essential to satisfy rumen microbial protein synthesis. Adequate dietary S also enhances utilization of nonprotein nitrogen in the rumen (Garrigus 1970, Knight 1985).

Dietary S requirements of ruminants are influenced greatly by the population of microorganisms in the rumen. Sulfur entering the rumen is usually in inorganic and/or organic forms of sulfate, sulfite and sulfide (Fig.1). A variety of S containing compounds (both organic and inorganic) are reduced in the rumen to sulfites and sulfides (Lewis 1954). Normally, sulfide is used for the synthesis of microbial amino acids. However, excess sulfide is absorbed from the rumen. In the tissues, sulfide is oxidized to sulfite which is further oxidized to sulfate. Sulfate is recycled to the rumen via saliva.

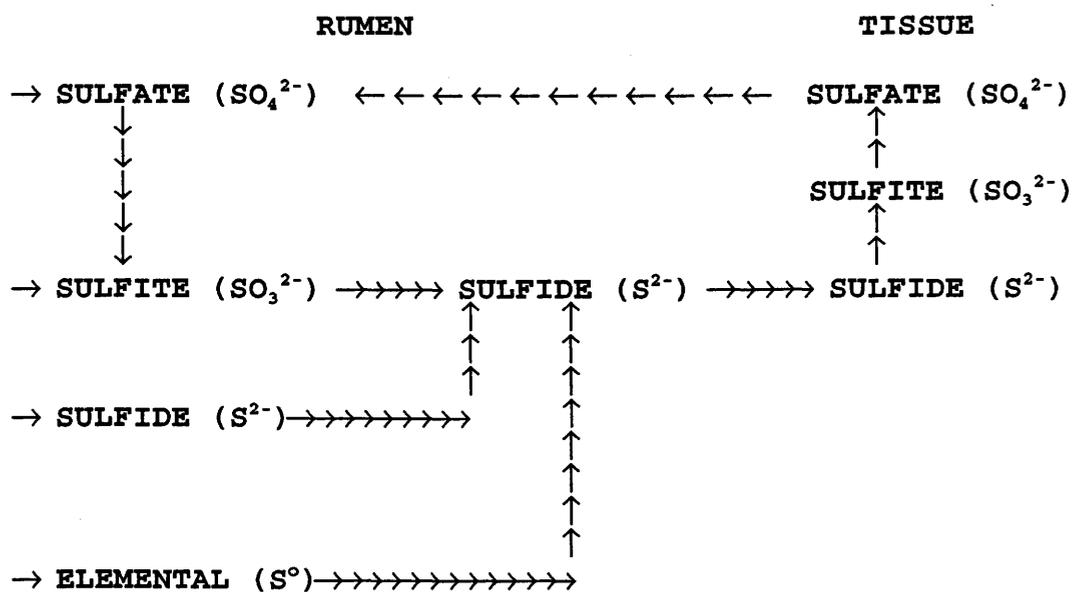


Fig. 1. Schematic outline of S metabolism in ruminants. Sulfur containing compounds are reduced in the rumen to sulfides. Excess sulfide is absorbed from the rumen and oxidized to sulfite and sulfate in the tissues. Sulfate is recycled to the rumen via saliva.

Elemental S and sulfate are relatively non-toxic. However, sulfites and sulfides are toxic. Excess dietary sulfur may cause proliferation of sulfur reducing-bacteria in the rumen (Lewis 1954). If excess S enters the rumen, sulfides can accumulate in concentrations high enough to cause toxicity. The metabolism and toxicity of S will be elaborated on in detail in the ensuing sections.

#### **2.1.2.1.1 Rumen bacteria and S metabolism**

Rumen microorganisms can utilize inorganic S to synthesize S-containing amino acids in the production of microbial protein. Loosli et al. (1949) first reported that sulfate-sulfur was incorporated into cysteine and methionine of microbial protein. The latter authors also reported that these specific amino acids were subsequently incorporated into the proteins in milk and wool produced by the animal. Others reported similar findings (Block et al. 1951, Hale and Garrigus 1953).

Metabolism of bacteria in the rumen is influenced by S, with both form and quantity being important factors (Lewis 1954). Although there is a high level of interaction between these criteria, quantity is more critical. Sulfur concentration in the diet influences the population of S-reducing bacteria in the rumen. The S reducing bacteria may be divided into two groups of organisms. The bacteria reducing S for nutritional purposes represent an assimilatory group, whereas reduction of S, as an effect of electron transfer in the respiratory chain, characterizes a dissimilatory group (Huxtable 1986). Sulfur-reducing bacteria

increase in number when the concentration of dietary S increases (Lewis 1954). An elevated concentration of sulfate-reducing bacteria was found in the rumen content in sheep fed sulfate-containing diets, suggesting that the dissimilatory sulfate reducing group makes a major contribution to the total sulfate reduced in the rumen of sheep (Huisingh et al. 1974). A similar opinion was expressed by Howard and Hungate (1976).

#### **2.1.2.1.2 Biochemistry of S in the rumen**

A variety of S containing compounds are reduced in the rumen to sulfide (Lewis 1954). A transient accumulation of sulfide occurs in the rumen under a variety of feeding regimens (Spais et al., 1968). The optimum pH for the reduction of sulfate is 6.5 (Anderson 1956), but the rate is not very sensitive to pH changes. Sulfide produced by rumen microorganisms can be utilized to synthesize S-containing amino acids in the production of microbial protein. A few rumen bacteria require amino acids which contain S for growth (Pittman and Bryant, 1964), although there is a consensus that sulfide is a major source of S for bacterial protein synthesis and amino acids containing S are synthesized de novo. Gawthorne and Nader (1976) found in sheep that only 53-57% of the S in microbial protein originated from the sulfide pool.

#### **2.1.2.1.3 Absorption of S from the rumen**

Anderson (1956) suggested that sulfide is directly absorbed from the rumen. This has been confirmed by other workers. Bray (1969a,b) showed that there was

very little absorption of sulfate-S, whereas the absorption of sulfide-S was very rapid.

#### **2.1.2.1.4 Recycling of S to the rumen**

The possible routes for S recycling to the gastrointestinal tract are: a) desquamation of ruminal epithelium, b) entry through the rumen or intestinal wall, c) entry through salivary secretions. Saliva is probably the major source of recycled S to the rumen of sheep and cattle. Total S in parotid saliva ranges from 1.4 to 12.5 mg/L (Church 1988). Most of the S entering the rumen with saliva is in a form of sulfate (Bray and Hemsley 1969, Kennedy et al. 1976, Kennedy and Milligan 1978). Rumen bacteria may derive a substantial part of S requirements from endogenous sources (Kennedy and Milligan 1978).

#### **2.1.2.2 In the intestines**

##### **2.1.2.2.1 Absorption and secretion of S**

Sulfate is absorbed from the small intestines (Bird and Moir 1971). Bird (1971) showed that sulfate infused into the duodenum and not absorbed in the small intestine may be reduced in the large intestine to hydrogen sulfide which is then absorbed. Sulfide resulting from microbial degradation of various S compounds in the hindgut is not extensively used for protein synthesis. Sulfur is present in the fluids secreted into the intestines. The combined biliary and pancreatic secretions may contribute 142-245 mg S per day (Bird 1972a).

##### **2.1.2.3 Excretion of S**

Sulfur is excreted in the urine in the form of both organic and inorganic compounds. Sulfate is the major end product of S amino acid oxidation and inorganic S metabolism in mammalian tissue. Urinary excretion of sulfate represents a major route of S elimination from the body. Generally, only trace amounts of amino acids appear in urine, and ruminants, like monogastrics, excrete ester sulfates in the urine. Variations in S intake affect urinary S excretion (Bray and Hemsley 1969). Bird (1971) found that infusions of sodium sulfate into the rumen or duodenum increased the excretion of total S in forms of ester sulfate, and inorganic sulfate in urine.

The major portion of faecal S is organic (Bird 1971, Bird and Hume 1971) and is presumably derived from bacterial protein. The faecal excretion of total S increases with S intake and has been also shown to be related to intakes of organic matter and digestible organic matter (Kennedy 1974). Faecal S excretion by sheep may be increased when oral or ruminal supplements of sodium sulfate are given. Bray (1969a) reported faecal recovery of 12 and 19% of S from intravenous doses of S labelled  $\text{Na}_2\text{SO}_4$  and suggested that intravenously infused S-sulfate was secreted primarily into the post-ruminal tract in the form of sulfate esters or inorganic sulfate, and excreted as such in the faeces.

### **3.1 Sulfur toxicity**

#### **3.1.1 General toxicology of S**

Elemental S and sulfate are relatively non-toxic. The most common toxicities of S are associated with sulfide and sulfite.

### 3.1.1.1 Sulfite

The substantial portion of the toxicities attributed to sulfite involve a form of hypersensitivity. Reports on reactions to sulfite in the general medical literature most often involve asthmatics, indicating that the more severe response occurs in this group (Jamieson et al. 1985). Six fatalities allegedly associated with sulfited restaurant food were reported (FDA Report 1985). The symptoms of sulfite hypersensitivity which may involve multiple target organs include: nausea, bronchoconstriction, wheezing, dyspnea, stomach cramps, diarrhoea, tingling sensations, flushing, hypotension, cyanosis, shock, and loss of consciousness. Symptoms range in severity from mild discomfort to life-threatening episodes and death (Jamieson et al. 1985). Sulfite, being a strong nucleophilic anion, can react with a variety of humoral and cellular components and cause toxicity (Gunnison 1981).

The mechanism of sulfite hypersensitivity is not known. Impairment of sulfite oxidase may play a role in the hypersensitivity of some individuals (Jacobsen et al. 1984). One of the possible mechanisms may be based on the readiness of sulfite to react with disulfide bonds to form S-sulfonates by a process termed sulfitolysis (Cecil 1963). Such engagement of disulfide bonds in low molecular weight compounds (e.g., cystine or glutathione, lipoate) or proteins may hypothetically lead to impairment of cellular processes. However, the physiological importance of sulfitolysis reactions is unknown. The possibility of involvement of the immune system in sulfite hypersensitivity must be considered.

The presence of bisulfite caused the release of histamine from the peripheral leucocytes of sulfite-hypersensitive individuals (Twarog and Leung 1982).

Sulfite may be an important factor in activation of potentially carcinogenic compounds. It is considered a co-carcinogen for benzopyrene in the respiratory tract of rats and hamsters (Leung et al. 1989). The sulfite co-carcinogenic effect may be via altering detoxification or facilitating the reaction of a carcinogen with DNA (Leung et al. 1989). Sulfite caused DNA damage in the presence of cobalt, copper and manganese, although sulfite alone or metal ion alone did not (Kawanishi et al. 1989). Depletion of glutathione may play a role in enhancement of mutagenicity (Reed et al. 1990). Maternal toxicity, as evidenced by decreased body weight gain and decreased food consumption was observed in rats exposed to sodium sulfide, but no clinical signs of toxicity were observed. A significant reduction in the fetal body weight of both sexes was observed, but external, skeletal and internal fetal malformations were not observed (Itami et al. 1989). These authors concluded that sodium sulfite produced signs of fetal toxicity but no evidence of teratogenicity.

The potential toxicity of sulfite-derived free radicals should be considered. A variety of S-containing free radicals have been described (Lunazzi and Pedulli 1985). Sulfite-derived radicals can be formed through catalytic reaction of peroxidases and are postulated to cause lipid peroxidation and damage to the biological membrane (Mottley and Mason 1988). There is a possibility that sulfite-derived radicals can react with various macromolecules and these adducts

can form haptens, thus inducing immune response. In one immune disorder affecting the CNS, the principal antigenic glycolipid has been identified as sulfite-3-glucuronyl paragloboside. The sulfited glucuronate was found to be an important part of the epitope (Quarles et al. 1986).

#### 3.1.1.1.1 Metabolism

Sulfite is generated in the body by normal catabolic processing of S-containing amino acids and other S-containing compounds (Cooper 1983). A major source of endogenous sulfite is cysteine. Formation of sulfite appears to be initiated by the oxidation of cysteine to cysteine sulfinic acid. The enzyme responsible, cysteine dioxygenase, is found in the cytoplasm and utilizes molecular oxygen which is incorporated into the product (Lombardini et al. 1969). In mitochondria, cysteine-sulfinic acid undergoes transamination with 2-ketoglutarate or oxaloacetate to yield 3-sulfinylpyruvate which undergoes spontaneous desulfination. Sulfur dioxide ( $\text{SO}_2$ ) formed in the latter reaction undergoes hydration and proton dissociation to form sulfite. This pathway accounts for most of the endogenous sulfite produced in the body. Sulfite can also be formed during the metabolism of hypotaurine. Hypotaurine is produced by decarboxylation of cysteine sulfinic acid (Spears and Martin 1982) or by oxidation of cystamine (Federici et al. 1980). Hypotaurine undergoes transamination with  $\alpha$ -ketoacids to form sulfinylacetaldehyde (Fellman and Roth 1982). The latter spontaneously desulfinate, yielding sulfite and acetaldehyde. Sulfite formation may also occur during the oxidation of hydrogen sulfide. Endogenous hydrogen

sulfide is formed during transaminative catabolism of methionine (Benevenga 1984), but overall it is synthesized in small quantities (Stipanuk and Beck 1982). Since hydrogen sulfide is extremely toxic, it is unlikely to play a significant role in the toxic effects of endogenous sulfite. Cysteine therefore appears to be the predominant precursor of sulfite in vivo.

Although sulfite is generated in considerable quantities, intracellular steady-state concentrations of sulfite are low in normal individuals probably because of sulfite oxidase, a mitochondrial enzyme which catalyses the oxidation of sulfite to sulfate. Exogenous sulfite is metabolized like endogenous sulfite. Most of the exogenous sulfite is oxidized to sulfate, and a small part is converted to thiosulfate, S-sulfoalbumin, S-sulfogluthathione and S-sulfocysteine (Togawa et al. 1990). Nonenzymatic autoxidation of sulfite to sulfate can be catalyzed by compounds such as superoxide anion, dimethyl sulfoxide, and trace metals (Shroeter 1966, McCord and Frodovich 1969). However, in animal tissues oxidation of sulfite to sulfate occurs mainly by an enzymatic process. Most of the inorganic sulfate excreted in the urine is produced through enzymatic pathways (Lundquist et al. 1980). Oxidation of sulfite by sulfite oxidase is very efficient, even at low concentrations of the substrate (Rajagopalan 1980). This is of primary importance to maintain very low concentrations of sulfite in the body tissues. The enzyme sulfite oxidase is a molybdohemoprotein located in the intermembranous space of mitochondria (Rajagopalan 1980) and is found in most tissues of the body (MacLeod et al. 1961ab, Johnson et al. 1977). There is, however,

considerable variability of sulfite oxidase activity among species. Hepatic activity of sulfite oxidase in rats was 20 fold higher than that in humans (Johnson and Rajagopalan 1976ab). Sulfite oxidase is essential for normal development. Severe neurological abnormalities manifested by mental and physical retardation were associated with sulfite oxidase deficiency (Irreverre et al. 1967, Shih et al. 1977, Waddman et al. 1983). Progressive destruction of brain tissue was observed in a patient with sulfite oxidase deficiency (Brown et al. 1989).

### **3.1.1.2 Sulfide**

Symptoms of sulfide toxicity depend greatly on the chemical form and route of exposure. Hydrogen sulfide is an irritant. Pulmonary edema occurred in various species after exposure to hydrogen sulfide (US-NRC 1979). The harmful effects of hydrogen sulfide on the nervous system structure and biochemistry have also been reported. Necrosis of the cerebral cortex, reduction in Purkinje cells of the cerebellar cortex, and focal gliosis were observed in rhesus monkeys exposed to 500 ppm of hydrogen sulfide (Lund and Wieland 1966). Hence, the brain may be a target organ of hydrogen sulfide toxicity. Warenycia et al. (1989) demonstrated increased net uptake and selective accumulation of sulfide in the brainstem of rats exposed to hydrogen sulfide. However, since this compound naturally accumulates in many tissues including the brain after death, the evaluation of specific sites of toxicity may be difficult. Sulfide concentrations in the blood, liver, and kidneys of rats increased in both the exposed and non exposed groups, depending on the lapse of time after death. On the other hand,

the lung, brain, and muscle showed little or no change in sulfide concentration (Nagata et al. 1990).

Hydrogen sulfide has the potential to reduce disulfide bonds in proteins (Smith and Abanat 1966). Reduction of disulfide bounds in proteins has been suggested as an additional toxic mechanism whereby enzyme function could be altered. Oxidized glutathione (GSSG) was found to be protective against the toxic effects of sulfide, while reduced glutathione was not (Smith and Abanat 1966). The mechanism of the protection by glutathione was presumed to be through scavenging of hydrosulfide by the disulfide linkage of oxidized glutathione, thereby preventing the reaction of sulfide with other more critical thiol groups e.g. present in enzymatic sites, hormones, etc.

The reaction of sulfide with proteins has been implicated in the toxic mechanism of this compound. The biochemical mechanism of toxicity has been regarded to involve inhibition of mitochondrial electron transport (Smith and Gosselin 1979), via a selective reaction with cytochrome  $aa_3$  (Nicholls 1975a, Wever et al. 1975, Peterson 1977). Reaction of sulfide with metals present in vital metalloenzymes such as cytochrome oxidase is a probable mechanism of toxicity. Under physiological conditions sulfide would block the respiratory chain primarily by inhibiting cytochrome c oxidase (Nicholls 1975b). Such a biochemical impairment would lead to functional (histotoxic) hypoxia in the lung tissues (Khan et al. 1990). Studies in vitro with rat lung mitochondria showed that low concentrations of sulfide caused selective inhibition of cytochrome c

oxidase activity (Khan et al. 1990). This effect was reversed upon removal of sulfide either by washing or by oxidation with methemoglobin.

There is a possibility of formation of thiosulfate and sulfide radical anions from the reactions of hydrogen peroxide with thiosulfate and sulfide ions respectively (Ozawa and Hanaki 1990). The latter authors suggested that these radical anions may be important in the toxicity of sulfide ion in living cells.

The carcinogenic, teratogenic, and reproductive effects of sulfides have not been studied extensively. However, such an effect should be considered. A recent study indicates that exposure to low levels of hydrogen sulfide does not alter reproductive parameters (Hayden et al. 1990a), although some minor metabolic alterations were noted. Blood glucose was elevated in maternal blood on day 21 postpartum upon exposure to low levels of hydrogen sulfide. This increase in glucose was accompanied by a decrease in serum triglyceride in the pups and in the dams on d 21 postpartum (Hayden et al. 1990b). Hydrogen sulfide may produce deleterious effects on the developing central nervous system. Exposure of pregnant rats to carbon disulfide and/or hydrogen sulfide at levels of exposure associated with maternal toxicity caused fetal toxicity (Saillenfait et al. 1989). A preliminary study of developing cerebellar cells of animals chronically exposed to low concentrations of hydrogen sulfide during perinatal development indicates that some structures are at risk of severe underdevelopment (Hannah and Roth 1991). There is some indication that salts of sulfide with some metals may have a carcinogenic effect (Zhong et al. 1990).

### 3.1.1.2.1 Metabolism

The metabolism of sulfide can be divided into three distinct pathways: (1) oxidation to sulfate; (2) methylation; (3) reaction with metallo- or disulfide-containing proteins. The first two metabolic pathways usually can be regarded as detoxification routes, whereas the reaction of hydrogen sulfide with essential proteins is largely responsible for its toxic action.

Sulfate is the end product of sulfide entering tissues. The majority of [<sup>35</sup>]-sodium sulfide administered via common routes was excreted in the urine as sulfate in the first 6 to 12 hr after dosing (Curtis et al. 1972). Oxidation of sulfide in the body is primarily an enzymatic process. The sulfide oxidase activity was found to be associated preferentially with the mitochondrial fraction. The sulfite, which is an intermediate, was suggested to be catalytically converted to sulfate by sulfite oxidase, an enzyme which has been identified in both liver and kidney tissue (MacLeod et al. 1961a,b).

Following sequential oxidation, sulfide is primarily excreted as either free sulfate or conjugated sulfate directly in the urine. However, the mechanism of the oxidative metabolism of sulfide to sulfate has not been completely elucidated. Another method of sulfide disposal in mammalian tissues is methylation to methanethiol and dimethylsulfide (Weisiger and Jakoby 1980). Interaction of sulfides with hemoglobin to form "sulfhemoglobin" has been reported. However, disposal of sulfide via sulfhemoglobin does not appear to play a significant role

in the acute toxicity of sulfides since it is not found in meaningful concentrations in vivo (US-NRC 1979).

### **3.1.2 Sulfur toxicity in ruminants**

#### **3.1.2.1 Sources of S toxicity**

##### **3.1.2.1.1 Excess S in feeds**

High to excessive S concentrations in some plants occur naturally and can increase under a variety of soil management conditions (Boila et al. 1987). High concentrations of S are inherently present in a number of commonly used feedstuffs (Table 1), and subsequently excessive S content can be expected in the rations based on these ingredients.

The contribution of environmental S includes inherent and acquired factors. The inherent factors are those which originate from geological configuration (composition of soil and water), whereas acquired factors may originate from industrial sources (e.g. power plants, factories, smelters, etc.) and intensive farming practices (e.g. fertilisers, waste products of animal industry, etc.). These factors can inadvertently affect the composition of the diet and influence the metabolism of an animal.

In areas where S content of soil and/or water is high the uptake of S by plants may be significant (Boila et al. 1987). Further, constantly increasing emissions of S products from industrial sources make, no doubt, a significant contribution. The pollution with airborne S compounds presents perhaps a more discrete problem.

Table 1. Feedstuffs containing high concentrations of S

Feed	Sulfur content % (DM)
alfalfa	0.48%
corn cobs	0.40%
extracted cotton seeds	0.34-0.56%
mangel beets	0.63%
sugar beets and their byproducts	0.22-0.54%
soybean meal	0.49%
molasses	0.40-0.61%
oat hay	0.25%
rape seeds mechanically extracted	0.50%
ryegrass	0.30%
sesame seeds mechanically extracted	0.35%
soybean and soybean byproducts	0.26-0.48%
sweetclover hay	0.47%
turnip	0.43%
yeasts	0.45-0.62%
wheatgrass	0.47%
dehydrated whey	1.12-1.15%
brewers dried grains	0.32%

According to NAS-NRC (1984)

Plants can absorb S directly from the atmosphere and the foliage may be covered with S containing particles.

#### 3.1.2.1.2 Excess S in water

Numerous studies indicate that S in the water, even at moderately excessive levels, may increase the total dietary S to a toxic level (Smart et al. 1986, Boila 1988, Olkowski et al. 1991a, Beke and Hironaka 1991). However, S in the water has been often ignored in the calculation of the total dietary intake of S in ruminants.

Water intake by cattle depends on ambient temperature and can vary from 3 L kg<sup>-1</sup> DM at 5° C up to 8 L kg<sup>-1</sup> DM at 32° C (National Research Council 1984). Hence, the consumption of water containing 1000 ppm of sulfate can increase the dietary intake of S by approximately 0.1 to 0.27 %.

Dietary S at a level above 0.3 - 0.4% may cause toxic effects (Kandylis 1984, NAC-NRC 1984). Considering that the S content in common ruminant's rations is in a range 0.15 to 0.20% of DM, the consumption of water containing 1000 ppm of sulfate can increase the dietary S to a toxic level. According to Canadian standards, drinking water containing 1000 ppm or more sulfate (333.33 mg S/L) is not recommended for any livestock (Canadian Water Quality Guidelines 1987). However, it seems rather futile to set the limit for S content in the drinking water for ruminants, without also considering the rate of water consumption and S content in the feed. It is important to stress that these factors may be extremely variable.

### 3.1.2.1.3 Mineral supplements

Most of the common feed grade mineral supplements (other than sulfate salts) of calcium, cobalt, copper, magnesium, potassium, sodium, iron, and zinc, usually contain 0.2 to 2.2% of S as impurities, whereas sulfate salts of these minerals typically contain 10 to 24% of S (NAS-NRC 1984). Under normal dietary conditions the contribution of S from mineral supplements is relatively small, however, if the dietary S obtained from feeds and water is already excessive, the contribution of mineral supplements may be significant.

### 3.1.2.1.4 Nutritional errors

Toxicities of S resulting from nutritional errors are relatively rare. Elemental S was often fed to livestock as a tonic and to control external parasites. Some feedlots used S to restrict the consumption of feed (Raisbeck 1982). Accidental, acute sulfur toxicity in cattle has become considerably less common in recent years, although sporadic cases are still being reported (Gunn et al. 1987, Short and Edwards 1989). The nutritional problems are frequently associated with secondary metabolic effects. Nutritional errors ought to be also considered in the context of proper balance of micronutrients. Marginally adequate levels of selenium (Pope et al. 1979) or copper (Gooneratne et al. 1989) are likely to exacerbate the detrimental effect of even moderately high dietary S. The effect of dietary S on copper may be more pronounced in the situation where the content of other divalent metals in the ration is excessive (Gooneratne et al. 1989c).

### **3.1.2.2 General aspects of S toxicity in ruminants**

The mechanism of toxicity of sulfites and sulfides has not been studied extensively in ruminants. The basic responses (oxidation to sulfate, methylation, reaction with thiol groups, etc) are assumed to be qualitatively similar among animals, however, there may be quantitative differences. For example, hepatic activity of sulfite oxidase found in rats was 20 fold higher than that in humans (Johnson and Rajagopalan 1976ab). It would be of interest to study the toxicological aspect of the kinetics of the disposal mechanisms for sulfides and sulfites in ruminant species.

In ruminants, relative to monogastric species, sulfides and sulfites are generated in considerable quantities and their elimination may be of primary toxicological importance. Most reports relate to either acute veterinary problems or to more subtle nutritional problems.

#### **3.1.2.2.1 Acute toxicity problems**

In small doses S is relatively non-toxic but excessive doses can cause fatal gastroenteritis and dehydration (Julian and Harrison 1975). Most acute problems occur from overdosing. Continuous feeding of S at the rate of 7 g per day can be fatal to adult sheep (White 1964). Accidental, acute sulfur toxicity in cattle has become considerably less common in recent years, although sporadic cases of sulfur toxicoses are still being reported (Short and Edwards 1989). Environmental factors causing S toxicity may be of increasing importance. Animals confined to industrial areas, near smelters, natural gas facilities, power plants or factories

burning large amounts of coal may become poisoned by S dioxide. Exposure of grazing animals to a sulfur dioxide concentration of 500 ppm for 1 h is dangerous (Hatch 1977). Animals also may become poisoned by S from environmental pollution when exposed to S dust, S dioxide, and hydrogen sulfide. Janowski and Chmielowiec (1981) reported poisoning of cattle exposed to S dust, S dioxide, and hydrogen sulfide within 1 km of a S mine. Poisoning was associated with lesions of the respiratory and digestive systems.

A slatted floor system of manure disposal, if functioning imperfectly, may liberate hydrogen sulfide gas in hazardous concentrations. Sudden exposure to hydrogen sulfide concentrations of 400 ppm may be fatal (Hatch 1977).

The minimum lethal dose of sodium metabisulfite in sheep is 2.25 g/kg of body weight (Nikolaev and Dzhidzheva, 1973), and signs of toxicity which included restlessness, feed refusal, ruminal atony, rapid pulse and respiration, cyanosis, and death.

Sulfur poisoning following the excessive oral administration of elemental S resulting in fatalities was reported in cattle (Harvey 1924, Coghlin 1944, McFarlane 1952, Julian and Harrison 1975, Gunn et al. 1987) and sheep (White 1964). In the reported cases mentioned above, toxicity was secondary to excessive administration of elemental S as an oral therapeutic.

Clinical signs associated with acute S toxicity are characterized by dullness, abdominal pain, muscle twitching, black diarrhoea and a strong odor of hydrogen sulfide on the breath. Dehydration is severe and the animals soon become

recumbent and dyspnoeic, develop convulsions and die in a coma. At necropsy the lungs are congested and edematous, the liver is pale, the kidneys congested and black in color and there is severe gastroenteritis with peritoneal effusion. Petechial hemorrhages have been observed to occur extensively in all organs and in musculature (White 1964). Acute renal lesions were reported in cattle poisoned with elemental S (Gunn et al. 1987) and sheep poisoned with S or with DL-methionine (Doyle and Adams 1980).

The clinical abnormalities seen with excess elemental S ingestion appear to result from the products of S metabolism in the rumen, as elemental S itself is relatively nontoxic. Sulfur is rapidly reduced in the rumen to sulfide and excessive sulfide production within the rumen may result in a build-up of toxic hydrogen sulfide gas (Knight 1985). It has been suggested that toxicity of hydrogen sulfide in ruminants is apparently due to the eructation and inhalation of hydrogen sulfide produced in the rumen (Knight 1985).

The disposal of hydrogen sulfide has not been investigated in the ruminant species. In general terms the metabolism of hydrogen sulfide may be similar to that in other species (oxidation to sulfate, methylation to methanethiol and dimethylsulfide, reaction with thiol groups). However, considering that ruminants were constantly required to metabolize sulfide absorbed from the rumen sulfide during evolutionary adaptation, it is quite probable that this species developed some additional (perhaps more efficient) systems of sulfide disposal.

While the toxicity of hydrogen sulfide is unquestionable, it is not clear

whether or not the amounts of hydrogen sulfide liberated in the rumen are sufficient to cause toxic effects. Hydrogen sulfide in solution is relatively non-toxic (Goodman and Gilman 1970). The sulfide portion produces cathartic action in the intestines. The liver itself is very efficient in detoxifying sulfide through the action of a sulfide oxidase system (Anderson 1956). Hence, it is unlikely that much free sulfide would reach the brain after being absorbed from the rumen into the portal system (Bird 1972). Bird (1972) stated that "the direct and shorter route to the heart and brain afforded by the inspiration of hydrogen sulfide and transfer into the pulmonary vein effectively bypasses the liver and enables hydrogen sulfide to exert its toxic effect on the respiratory-circulatory systems". However, Julian and Harrison (1975) found lesions only in the digestive tract of sulfur poisoned cattle. Irritation of the respiratory and gastrointestinal mucosa (Martin and Willoughby 1971, Gilman et al. 1985) is consistent with the findings of Gunn et al. (1987) in cattle poisoned with elemental S. Since the liver efficiently removes sulfide absorbed into the portal vasculature, toxicity of sulfide in ruminants due to the eructation and inhalation of hydrogen sulfide produced in the rumen cannot be excluded. Irritation of the respiratory epithelium would be consistent with inhalation of hydrogen sulfide gas during eructation. Bray (1969b) found that when 1.01 g of S as sodium sulfide was infused into the rumen, hydrogen sulfide could be smelled on the animal's breath. However, the very high levels of sulfide in the rumen in the work of Anderson (1956) did not result in any toxic symptoms, nor did the addition of sulfide to give rumen

concentrations of 330  $\mu\text{g}$  sulfide/ml. Bray (1969b), however, observed respiratory difficulty in sheep when sulfide levels of approximately 330 and 226  $\mu\text{g}/\text{ml}$  were added to buffers replacing rumen contents, and hydrogen sulfide could be smelled on the animals' breath in 10 to 15 min.

Hence, direct involvement of hydrogen sulfide in the pathogenesis of S toxicity in ruminants remains to be investigated more thoroughly. On the other hand, other potential pathways should be considered. Methylation of sulfide results in products which are less toxic than hydrogen sulfide. Methanethiol is lethal to rats at 10,000 ppm (Windholz 1983). Thus, methylated products do present a toxicity problem. Elevated levels of sulfhemoglobin were observed in cattle drinking high sulfate water (Weeth and Hunter 1971). Sulfide absorbed from the rumen may be bound by oxygenated hemoglobin. However, Weeth and Hunter (1971) did not observe any evidence of hypoxia or changes in hematocrit in heifers drinking water containing 5000 ppm sodium sulfate. Formation of sulfhemoglobin probably is not significant in overt toxicity of S in ruminants. The potential involvement of sulfite in the etiology of S toxicity in ruminants should receive more attention. This compound is generated in the rumen during the process of reduction of sulfate to sulfide (Lewis 1954) and systemically during the oxidation of sulfide to sulfate. In view of the recycling of S products between the rumen and the host, there is a potential for sustained presence of sulfite both in the rumen and tissues.

### 3.1.2.2 Nutritional problems

More subtle problems associated with S toxicity occur as a variety of nutritional problems. Detrimental effects of S-containing compounds were noted by many workers. However, the findings on this subject reported in the literature are very inconsistent. Dietary levels of 0.3% and more have a detrimental effect (Kandyliis 1984). It appears that there is a very narrow margin between requirements and toxicity. The dietary requirement for sheep is 0.14 to 0.26% S (NAS-NRC 1985). For steers the requirement is 0.1% up to 0.15% S and for lactating cows 0.20% S (NAS-NRC 1984). Often, the S requirement for ruminants is expressed on the basis of the N to S ratio. This ratio is most often given as 15:1 for cattle and (10-12):1 for sheep.

High levels of calcium sulfate (gypsum) are known to reduce feed intake of ruminants (Christensen et al. 1947). This principle has been used as an effective means for regulating intake of pasture supplements for livestock. Johnson et al. (1968) reported that the addition of 0.5% S as calcium sulfate reduced the daily gains of lambs. Bouchard and Conrad (1973b) reported reduced feed intake by dairy cows when calcium sulfate was used to increase dietary S to 0.3% or more. Thompson et al. (1972) reported reduced feedlot gain for steers supplemented with elemental S to produce a 5:1 N:S ratio. Albert et al. (1956) reported that elemental S or sodium sulfate added at a level above 0.4% of total S reduced the performance of lambs. On the other hand, some researchers have observed no harmful effects from feeding relatively higher levels of S. For example, Chalupa

et al. (1971) fed elemental S at levels up to 1.72% in a purified diet for Holstein calves with no detrimental effects. Similar findings were reported by Slyter et al. (1988). In the other study neither sodium sulfate nor elemental S (0.62% and 0.56% S, respectively) in the diet of steers produced harmful effects (Chalupa et al. 1971). However, Bouchard and Conrad (1973ab) showed that relatively lower levels (0.20-0.24% added S) of sulfate resulted in reduced performance. Bird (1972b) found that ruminal infusion of 6 g S as sodium sulfate per day resulted in complete inappetence. On the other hand, no toxic effects of S were observed by Gawthorne and Nader (1976) where 10 g of sodium sulfate/day were infused continuously into the rumen of sheep.

Kennedy et al. (1971) found no depression of starch digestion by ruminal microorganisms in vitro when S up to 11 mg/ml was added (as sodium sulfate), and no effects on growth in vitro of ruminal microbes was observed in studies by Bird (1972b) when sulfide was up to 1.5 mg/ml. Dougherty et al. (1965) suggested that generation of large quantities of hydrogen sulfide in the rumen depresses ruminal motility and causes severe nervous and respiratory distress if it is absorbed into the lungs during eructation. Bird (1972b) showed that single ruminal infusions of sulfide (0.94 g S) in solution ( $\text{Na}_2\text{S} - \text{H}_2\text{S}$ ) resulted in temporary respiratory distress and collapse of sheep. Sulfide toxicosis, however, apparently has not been associated with feeding of supplemental sulfate to ruminants (Boyazoglu et al. 1967) or with high sulfates in drinking waters (Peirce, 1960). In view of the latter studies, there is an apparent paradox. Sulfate-S is

more rapidly reduced to sulfide than elemental S. Radioactive S isotope studies showed that radioactivity from sodium sulfate appeared in the plasma rapidly, reaching peak concentration within 2 h, whereas that from elemental S or methionine appeared in the greatest amounts in the plasma 42 to 54 hours after dosing. However, most of the acute S poisonings are from ingestion of elemental S. This can not account for differences in the clinical picture seen in elemental S toxicity.

### **3.1.2.3 Sulfur-nutrient interactions in ruminants**

Interactions of S with nutrients account for a very specific kind of toxic effect. A number of these interactions have been extensively studied. The most investigated, and subsequently best understood are S-copper-molybdenum interactions.

#### **3.1.2.3.1 Sulfur-Copper-Molybdenum interactions**

Huisingh et al. (1973) proposed that copper becomes unavailable via two routes: 1) the formation of cupric molybdate which is absorbed and excreted rendering both copper and molybdate less available; and 2) the formation of an insoluble cupric sulfide in the rumen, intestine, or tissues. Suttle (1974) showed detrimental effects of excess S on Cu metabolism. It has been suggested that Mo apparently exerts its limiting effect on Cu retention in the presence of inorganic sulfate and neither Mo nor sulfate alone interferes with Cu retention (Whanger 1972). Copper and molybdenum can form a complex molecule known as cupric molybdate, although these complexes are not known in biological systems.

However, interactions of copper and S do occur in the gastrointestinal tract of ruminants. Suttle (1974) postulated that S (organic and inorganic) interferes with Cu retention, presumably because of the formation of insoluble CuS at sites beyond the rumen. A metabolic interrelation between S, molybdenum and copper was observed and, to explain the mechanism of these interactions, the formation of an insoluble  $\text{CuMoS}_4$  complex was proposed by Dick et al. (1975). Now, it is commonly accepted that decreased bioavailability of copper is due to formation of thiomolybdates (Allen and Gawthorne 1987, Gooneratne et al. 1989a, Golfman and Boila 1990).

Molybdenum inhibits the reduction of sulfate to sulfide *in vitro* (Huisingsh and Matrone 1972) and *in vivo* (Gawthorne and Nader 1976). However, it has been reported that the supplementation of Mo increases *in vivo* sulfide concentrations in the rumen of sheep (Mills 1960). On the other hand, Huisingsh et al. (1975) found that dietary sodium molybdate significantly inhibited the production of sulfide from sulfate, but enhanced the production of sulfide from methionine.

#### **3.1.2.3.1.1 Sulfur-induced copper deficiency**

In ruminants, excess dietary S can influence the metabolism of Cu (Suttle 1974, Christensen et al. 1984, Smart et al. 1986). Excess dietary Mo in the presence of high S results in the formation of thiomolybdates (TM) that further reduce the availability of Cu not only in the GI tract but also systemically (Price et al. 1987, Allen and Gawthorne 1987, Gooneratne et al. 1989c). A persistent decrease in the concentration of soluble Cu in the plasma was observed in sheep treated with TM

(Kincaid and White 1988). Smart et al. (1986) have shown that high S intake through drinking water can deplete liver Cu in cattle.

One of the more extensively studied effects of Cu deficiency is its effect on the immune function. Copper may be of importance in the antigen-antibody interaction (Philips et al. 1982). Impaired neutrophil candidacidal activity has been reported in S induced Cu deficiency in sheep and cattle (Jones and Suttle 1981). Suppression of both candidacidal and phagocytic ability of polymorphonuclear neutrophils from cattle has been recorded in high Mo or iron induced Cu deficiency (Boyne and Arthur 1986).

#### **3.1.2.3.2 Sulfur and other minerals**

Ganther and Bauman (1962) reported that the addition of sulfate increased the excretion of selenium in the urine. Higher blood selenium was observed in sheep fed a sulfur deficient diet than in those with sulfate added (Pope et al. 1968). Using everted sacs of sheep ileum, Turner et al. (1990) showed that sulfate and thiosulfate inhibited the uptake of selenate. The possible involvement of sulfate in an increased incidence of muscular dystrophy was reported (Hintz and Hogue 1964). Muscular dystrophy has been reported in sheep fed high S diets (Ganther and Bauman 1972). However, other workers could find no evidence for sulfate contributing to white muscle disease (Whanger et al. 1969). Also Khan et al. (1987) did not observe significant changes in blood selenium concentration or enzyme activities in cattle fed excess S. According to Pope et al. (1979) the effect of dietary S may be reversed by an increased supplementation of selenium.

Hence, the effect of S may be of more importance in cases of marginal adequacy of selenium.

Goodrich and Tillman (1966) reported that the retention of both calcium and phosphorus was reduced by the addition of sulfate to lamb diets. The effect on calcium was suggested to be due to the formation of calcium sulfide in the rumen. The apparent absorption and retention of calcium and magnesium tended to be affected by S (Spears et al. 1985). According to Tucker et al. (1991) urinary excretion of Ca tends to be increased by S supplementation. Recent work of Golfman and Boila (1990) indicates that S, either alone or in a synergistic effect with molybdenum, can affect GI metabolism of zinc, manganese, magnesium and phosphorus.

### **3.1.2.3.3 Sulfur-thiamine-copper interactions**

#### **3.1.2.3.3.1 Chemistry of thiamine-copper complexes**

Formation of thiamine - Cu complexes in vitro has been described and analyzed by crystallography (Caira et al. 1974; Cramer et al. 1984; Archibong et al. 1989). However, formation of such complexes in vivo has not been reported. Copper promotes formation of thiamine disulfide in-vitro (Kobayashi 1972). Kawasaki et al. (1958) reported that thiothiamine is not cleaved by sulfite. This interaction remains to be elucidated in vivo. However, there is a potential for a protective mechanism of copper whereby conformational change to a thiazole open ring would protect the thiamine molecule against nucleophilic attack of sulfite.

### **3.1.2.3.3.2 Biological Interactions**

Cramer et al. (1984) suggested that thiamine may play a role in the bioregulation of Cu. It appears that there is a possibility for copper involvement in coenzyme-metal-apoenzyme interaction (Aoki and Yamazaki 1980). A biological interrelationship between dietary thiamine and Cu was shown in rats (Ellerson and Hilker 1985). Another study showed that in cattle with low blood concentrations of both Cu and thiamine, improvement in Cu status from deficient to adequate resulted in a marked increase in blood thiamine concentration (Gooneratne et al. 1989b). The above indicates that these two nutrients may interact in a reciprocal fashion. However, the molecular basis of this interaction is not known.

### **3.1.2.3.4 Sulfur-thiamine interactions**

#### **3.1.2.3.4.1 Chemistry**

The destructive effect of the sulfite ion on the thiamine molecule was known since the vitamin was isolated. The reaction was used to characterize the molecular formula of thiamine (Williams and Reuhle 1935). Kawasaki et al. (1958a,b) reported that the cleavage of thiamine by sulfite occurs in the absence and presence of oxygen. The latter authors showed the reaction proceeded more rapidly in a solution saturated with nitrogen than in one saturated with oxygen.

Leichter and Joslyn (1969) demonstrated that the cleavage of thiamine by sulfite is a first-order nucleophilic displacement. In this reaction the sulfur atom of sulfite is oxidized to form the sulfonic acid group and the nitrogen atom of the

thiazole moiety is reduced (Zoltewicz et al. 1982). In the overall reaction, 1 mole of thiamine reacts with 1 mole of sulfite to yield pyrimidine sulfonic acid and a hydroxyethyl-4-methylthiazole (Zoltewicz et al. 1982). It is noteworthy that the most active reaction takes place at pH 4.5 to 6.5 (Leichter and Joslyn 1969). These conditions of pH are low enough to allow the thiamine molecule to be protonated at the pyrimidine ring, making it more electrophilic. The first pKa value for thiamine is 5.8.

There is a potential for analogue synthesis. Fig. 2 shows the proposed reaction. The leaving group (thiazole moiety) is eliminated to generate a reactive electrophile that is then captured by a second nucleophile to give rise to a substitution product (Zoltewicz et al. 1982). It is suggested that the mechanisms of action of the sulfite ion is similar to that of the enzyme thiaminase I. Thiaminase I brings about nucleophilic substitution of thiamine with a number of heterocyclic compounds (Roberts and Boyd 1974).

#### **3.1.2.3.4.1 Thiamine structure-activity relationship**

Since there is a possibility of synthesis of thiamine metabolites in the rumen, it is important to briefly introduce the subject of biological activity in relation to structural alterations. Thiamine (Fig. 3) is a very specific molecule. Even minor alteration can cause a loss of biological activity. The carbon-2 position must be unsubstituted for biological activity. The high electron density required at the C-2 is produced by the thiazolium system. A substitution of the methyl group at position 4 by hydrogen or ethyl results in loss of activity.

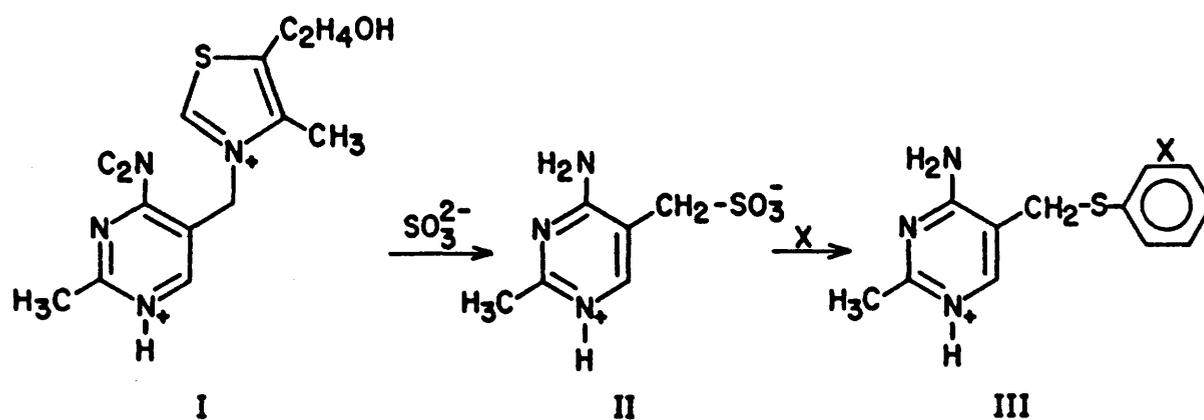


Fig. 2. Proposed reaction for formation of thiamine-like compounds catalyzed by sulfite ion. Thiamine molecule (I) is cleaved by sulfite ion to produce reactive intermediate (II) which can react with another heterocyclic compound (X) to produce a thiamine-like molecule (III).

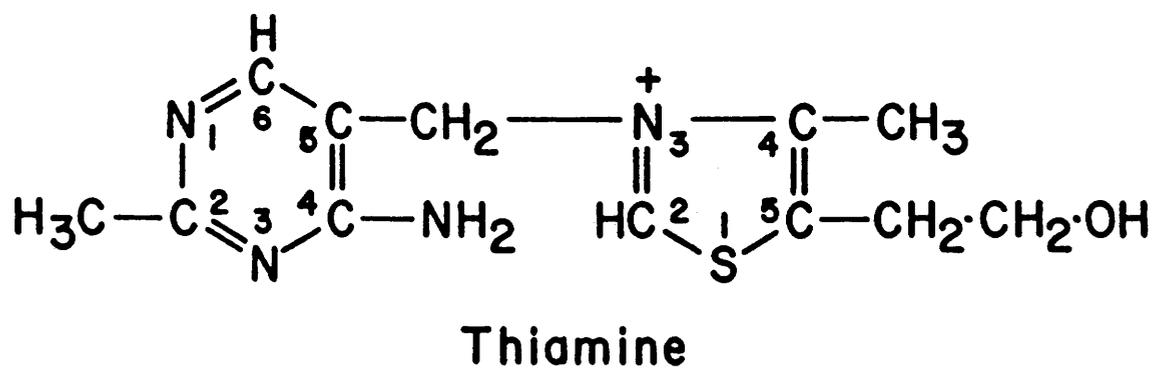


Fig. 3. Structural formula of thiamine molecule.

The methylene group between the two rings is important for maximum activity. If this group is eliminated or substituted, the activity is reduced drastically (e.g. if methylene is replaced by an ethylene group, there is 40% loss in activity). On the pyrimidine moiety the 2'-methyl group is of importance. The 2'-H homolog is inactive. The 2'-butyl compound is a potent antithiamine. Altering the methyl group of pyrimidine from the 2' to 6' position results in losses of activity. Substitution of the 4' amino group results in a compound (oxythiamine) with antithiamine activity. Other heterocyclic compounds do not provide biological activity. Substitutes for S in the thiazole moiety (oxazole, selenazole) cause loss of catalytic activity. Imidazolium compounds are considerably less active in catalytic tests. The vinyl analog (pyrithiamine) is a potent antagonist of thiamine. The compiled material in this section was based on the work of Rogers (1962).

#### **3.1.2.3.5 Sulfur induced thiamine deficiency**

Joslyn and Leichter (1968) observed that residual sulfite present in some preparations was largely responsible for the cleavage of thiamine during storage in aqueous suspensions, both at 25° C and when frozen at -15° C. Studdert and Labuc (1991) reported thiamine deficiency in cats and dogs associated with feeding meat preserved with sulfur dioxide.

Ruminants are considered to be self-sufficient in fulfilling the requirement for thiamine. However, thiamine deficiencies in both clinical and subclinical forms have been reported (Rammell and Hill 1986). The potential for thiamine destruction in the rumen does exist. Thiamine destroying activity in the rumen

of cattle, attributed to a thermostable factor of small molecular weight, was described by Quaghebeur et al. (1975). In the study of Loew et al. (1970), the thiamin-destroying activities in the rumen content of PEM cattle were substantially higher than in normal cattle, and these activities were mostly due to a thermolabile factor, since very little thiamin-destroying activity occurred in autoclaved rumen contents. Sulphur was not measured in these studies.

The involvement of sulfite in thiamine destroying activity in the rumen is highly probable. Decreased passage of thiamine to the duodenum was reported in cattle fed excess dietary sulfate (Goetsch and Owens 1987). Sulfate is reduced to sulfite and sulfide in the rumen (Lewis 1954). Notably, the preferable conditions for the reaction of sulfite and thiamine, i.e. an anaerobic and nitrogen rich environment (Kawasaki et al. 1958b) and a pH of 6.0 to 6.5 (Leichter and Joslyn 1969) are all present in the rumen. Furthermore, a number of possible co-substrates commonly found in the rumen (Brent and Bartley 1984) could create conditions favorable for synthesis of a thiamine-like compound (Zoltewicz et al. 1982), possibly having antimetabolite properties.

#### **3.1.2.3.5.1 Sulfur induced polioencephalomalacia**

Polioencephalomalacia (PEM), also known as cerebrocortical necrosis (CCN), was first identified as a distinct pathological entity in ruminants by Jensen et al. (1956) in the United States, and Terlecki and Markson (1961) in England. Although this condition has been recognized for more than three decades, its etiology is still not completely understood. The disease is one of the most

prevalent diseases of the central nervous system of cattle and sheep. It occurs in many parts of the world and is of considerable economic importance in areas of intensive production.

Historically, PEM (CCN) has been regarded as an effect of thiamine deficiency (Pill 1967, Daly 1968, Edwin et al. 1968, Edwin and Jackman 1973, Edwin et al. 1979). However, a variety of other factors such as cobalt deficiency (Hartley et al. 1962, MacPherson et al. 1976), selenium toxicity (Maag et al. 1960), molasses toxicity (Mella et al. 1976) and lead poisoning (Christian and Tryphonas 1971) have been associated with the development of malacic lesions. The occurrence of clinical signs of PEM has been documented in cattle exposed to a high concentration of sulfate in feed (Raisbeck 1982, Sadler et al. 1983, Gould et al. 1991) and in water (Harries 1987).

Morphologically, PEM is characterized by a focal necrosis of grey matter in the brain. The term polioencephalomalacia applies to softening of the cerebrocortical gray matter [(Gr.) polio = gray, encephalo = brain, malacia = softening]. The lesions are qualitatively similar in all cases of PEM, but it is not clear whether or not PEM has any specific common etiology (Sullivan 1985). In most instances, the affected animals respond to treatment with thiamine (Pill 1967, Daly 1968, Thomas 1986). This observation led many researchers to conclude that thiamine deficiency or disturbances in thiamine metabolism are implicated in the pathogenesis of this syndrome (Loew 1975, Edwin and Jackman 1982). Attempts to render ruminating calves or lambs thiamine-deficient by purely dietary means

have failed. However, Thornber et al. (1979) experimentally induced PEM in pre-ruminant lambs by dietary thiamine deprivation. The clinical, biochemical and neuropathological features of PEM have been reproduced in various species with the use of the thiamine analogue, amprolium (Loew and Dunlop 1972, Lilja 1973, Markson et al. 1974). Whether PEM is a simple thiamine deficiency disease or whether other factors are involved has not yet been resolved.

A number of PEM cases have been shown to be associated with the persistent activity of thiaminases in the gastrointestinal tract (Edwin and Jackman 1982). Two types of thiaminase with different modes of action have been described in the literature and micro-organisms producing each type have been isolated from the ruminal contents of PEM cases (Edwin and Jackman 1982). Thiaminase II is a hydrolase, where the catalytic reaction cleaves the thiamine molecule into its two moieties. On the other hand, thiaminase I is an alkyl transferase and requires a basic co-substrate for its activity, the co-substrate combining with the pyrimidinyl moiety of thiamine to form a new compound. A number of naturally occurring compounds (Brent and Bartley 1984) or synthetic anthelmintics, tranquilizers and antihistamines (Roberts and Boyd 1974) can serve as co-substrates for thiaminase I. The new compound could have structural similarities to anti-thiamine compounds. Support for the involvement of thiaminases in the aetiology of PEM has been provided by the work of Evans et al. (1975) who reproduced the disease by feeding bracken rhizomes to sheep.

A number of hypotheses were advanced in an attempt to explain the etiology of S-induced PEM. Raisbeck (1982) hypothesized that destruction of thiamine in the rumen by sulfite, which is the product of microbial sulfate reduction, may be the cause of S-induced PEM. This hypothesis is conceivable from a chemistry point of view (Leichter and Joslyn 1969, Zoltewicz et al. 1982). Goetsch and Owens (1987) attempted to explain their results by lowered pH in the rumen which may have caused release of bacterial thiaminase I (Brent and Bartley 1984). Inasmuch as this hypothesis is valid, the data of Goetsch and Owens (1987) do not support it. It is notable that the etiologies of PEM associated with thiaminase I and S induced PEM may have common features. The chemical reaction of sulfite with the thiamine molecule has a remarkable resemblance to the enzymatic reaction of thiaminase I (Zoltewicz et al. 1982).

In the recent work Gould et al. (1991) suggested that PEM can result from sulfide toxicosis following excess production of sulfide in the rumen. Unquestionably, sulfide is a potent toxicant of the CNS. However, it remains to be shown whether the amount of hydrogen sulfide gas liberated in the rumen is sufficient to cause necrotic lesions in the brain.

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## CHAPTER I. FIELD STUDIES

1. High sulfur related thiamine deficiency in cattle: A field study. S. Ravi Gooneratne, Andrzej A. Olkowski, Robert G. Klemmer, Gerald A. Kessler and David A. Christensen. *Can. Vet. J.* 1989, Vol. 30:139-146.
2. Association of sulfate - water and blood thiamine concentration in beef cattle: Field studies. Andrzej A. Olkowski, Colin G. Rousseaux, David A. Christensen. *Can. J. Anim. Sci.* 1991, Vol. 71:825-832.
3. Biochemical features in a herd of cattle with instances of polioencephalomalacia associated with drinking high sulfate water: A field study. Andrzej A. Olkowski and David A. Christensen.

**HIGH SULFUR RELATED THIAMINE DEFICIENCY IN CATTLE:  
A FIELD STUDY**

S. R. Gooneratne, A.A. Olkowski, R.G. Klemer, G.A. Kessler and D.A. Christensen. *Can. Vet. J.* 1989, Volume 30: 139-146.

**ABSTRACT**

Following development of polioencephalomalacia (PEM) in one of 105 cattle in a farm in southeastern Saskatchewan, a study was initiated to monitor thiamine and copper (Cu) status, and to evaluate interactive nutritional factors which may have been responsible for this occurrence. It was evident that a combination of high sulfur (S) and low Cu intake was responsible for the depletion of blood thiamine and plasma Cu. Supplementation with trace minerals alone resulted in a significant ( $p < 0.05$ ) improvement in both thiamine and Cu status of the herd. We recommend that herds exposed to high intakes of S be supplemented with Cu up to 50 mg/kg feed dry matter to alleviate potential deficiencies of thiamine and Cu.

## INTRODUCTION

Ruminants are not considered to require thiamine in their diet (1) since sufficient amounts are synthesized in the rumen to meet the demands of the mature animal (2). However, acute (3) and subclinical (4) thiamine deficiencies have been reported. There is little doubt that progressive thiamine deficiency is responsible for field cases of PEM which occur in ruminants (1). More recent reports have suggested a possible causal relationship between high intakes of sulfur (S) and PEM (5,6). Such incidents may occur during long-term consumption of high levels of S, and in those instances the whole herd is potentially at risk. Although, in some cases further intake of excessive amounts of S can be reduced, under practical farming conditions this is not always possible. This is especially so when water contains a high content of S, since the cost of water purification is exorbitant. Ruminant livestock in the Canadian Prairies can therefore be regarded as potentially at risk to PEM since water from deep aquifers on many farms contains high levels of S as sulfates (7,8).

Relevance of PEM induced by high levels of S in cattle in Canada is not known, nor is it known whether diets deficient in trace minerals increase the risk of the disease. Adverse effects of high intakes of S on metabolism of copper (Cu) in cattle have been reviewed (7). Although the role of S in decreasing the availability of dietary Cu in sheep is well established (9), the mechanisms underlying S-induced PEM are poorly understood.

The suggested role of S in the induction of both Cu (7) and thiamine (10) deficiency in cattle led us to suggest the existence of a Cu-S-thiamine interaction. In the rumen, S compounds are reduced to sulfides, and these combine readily with Cu to form insoluble Cu sulfides (9). Therefore we believed that in cattle fed moderate to high levels of S, a diet high in Cu alone might be beneficial in reducing the S load in the rumen. If our hypothesis is correct, a high concentration of Cu in the diet alone could be expected to improve indirectly the thiamine status of the herd. In this paper we report a field study carried out in southeastern Saskatchewan to test this hypothesis in cattle at a feedlot with a prior history of PEM. In this study we fed a custom-made trace-mineral supplement to animals of low to marginal thiamine and Cu status, and measured its impact on concentrations of thiamine and Cu in blood and plasma respectively.

## **HISTORY**

The farm involved was a 105 beef-cattle unit located in southeastern Saskatchewan. Calves of mixed breeding had been purchased through a local auction market in October 1986. They were separated into two weight classes and housed in two feedlot pens designated as North and South pens.

North pen: Housed 53 heavier calves. Their estimated ages at the time of purchase ranged from nine to eleven months of age and their mean body weight was 340 kg. Many calves in this pen were medium to large frame type and 44% were Simmental-breed crosses.

South pen: Housed the remaining 52 smaller calves. Their estimated ages at the time of purchase ranged from seven to nine months and their mean body weight was 260 kg. Only 18% of the animals in this pen were Simmental crosses.

Feeding and Management: All animals were fed a mixture of forage, barley and canola meal. Based on farmer's records the approximate daily feed intake by the steers in the north pen was 2.7 kg forage, 4.1 kg barley and 0.9 kg canola meal per animal. Feed analysis showed this diet to provide approximately 12.8% protein and 2.18 M cal/kg metabolizable energy. This was within National Academy of Sciences-National Research Council (NAS-NRC) (2) recommendations for this type of cattle. Smaller steers in the south pen were offered approximately 10% less feed than those in the north pen. Trace mineral and S analysis of the feed and water are shown in Table 1. The animals were also offered a free-choice commercial mineral (macro and micro) supplement.

The mineral supplement had not been analyzed previously, but according to the manufacturer's guaranteed analysis it should have supplied adequate amounts of both macro and micro-minerals (Table 2). Calves in both pens received water from a 40 m well drilled approximately two years previously. The water from this well had not been analyzed previously. Although the calves were consuming all the feed given to them, the owner had noticed that the animals were somewhat depressed and not growing at the expected rate.

Table 1. Trace mineral<sup>a</sup> and sulfur<sup>b</sup> concentrations in feed and deep well water.

Sample	Cu	Zn	Fe	Mn	Mo	Se	S
Forage	13.8	22.0	412.7	43.3	0.12	0.60	0.23
Barley	3.9	21.3	51.4	15.5	0.12	0.64	0.13
Canola meal	7.5	52.2	174.0	51.9	0.08	1.71	0.73
Deep well water (sampling 1) (Feb. 6, 1987)	0.01	0.04	0.02	0.007	0.18	0.006	437
Deep well water (sampling 2) (April 25, 1987)	0.004	0.04	0.02	0.005	0.05	0.006	442

<sup>a</sup> Cu, Zn, Mn, Mo and Se concentrations in feed and water are expressed as mg kg<sup>-1</sup> DM and mg L<sup>-1</sup> respectively.

<sup>b</sup> Sulfur concentrations in feed and water are expressed as % DM and mg L<sup>-1</sup> respectively.

Table 2. A comparison of manufacturer's guaranteed analysis and laboratory analysis of mineral mix fed prior to micropremix supplementation.

Units	Mineral	Guaranteed analysis by manufacturer <sup>a</sup>	Laboratory analysis of mineral mix <sup>b</sup>
%	Ca	14.0	11.40
	P	14.0	11.58
	Na	8.0	11.62
	S	c	0.57
mg kg <sup>-1</sup>	Cu	3,000	32
	Zn	5,025	332
	Fe	1,000	4941
	Mn	c	155
	Mo	c	0.5
	Se	c	0.32

<sup>a</sup> Guaranteed analysis printed on label. This feed was also guaranteed to contain: 1600 mg kg<sup>-1</sup> of iodine, 100 mg kg<sup>-1</sup> of cobalt, a minimum of 100,000 IU kg<sup>-1</sup> of vitamin A, 50,000 IU kg<sup>-1</sup> vitamin D<sub>3</sub>, and 100 IU kg<sup>-1</sup> of vitamin E.

<sup>b</sup> Analysis was carried out by the Feed Testing Laboratory, University of Saskatchewan.

<sup>c</sup> not available.

## CLINICAL FINDINGS

On February 4, 1987 the owner noticed that one animal (Simmental mix breed) in the north pen was scouring and walking with difficulty and sought veterinary advice (GAK). Examination of this steer (approximately 400 kg body wt) revealed a normal body temperature, passage of loose feces, and associated abdominal pain. The animal was treated with sulfa-methazine (Spanbolets, Norden Laboratories, Lincoln, Nebraska), sodium sulfosuccinate (Dioctol, rogar/STB, Montreal, Quebec) orally, and oxytetracycline (Liquamycin LP, rogar/STB, Montreal, Quebec) intravenously (IV) for coccidiosis, colic, and thromboembolic meningoencephalitis, respectively. The following day the animal was found recumbent and the veterinarian (GAK) was called again. The steer lay in lateral recumbency with legs extended. There was opisthotonos and animal was shivering. Examination revealed a normal temperature, rigidity of limbs, and loss of vision. At this time a tentative diagnosis of PEM was made and the steer was given thiamine hydrochloride (400 mg) (thiamine hydrochloride, MTC Pharmaceuticals, Cambridge, Ontario) and oxytetracycline (Liquamycin LP, rogar/STB, Montreal, Quebec) IV. By evening the animal showed some improvement but was unable to get up. The following morning the animal was able to rise and had regained coordination but was walking cautiously and appeared to be visually impaired. Administration of thiamine hydrochloride continued over the next four days, and approximately three weeks elapsed before this animal recovered completely. A blood sample was taken from this animal

on day 2 of treatment with thiamine (February 6). This animal became anorectic again on March 3, but recovered prior to any treatment. A blood sample taken from this animal on March 3 was analyzed for levels of Cu, zinc (Zn), iron (Fe) and thiamine. Another blood sample was taken from the same animal on April 25.

### **EXPERIMENTAL STUDY**

Since the sick animal responded readily to thiamine administration, diagnosis of PEM was presumptively confirmed on February 5, and a collaborative study was initiated to monitor the trace mineral [Cu, Zn, Fe] and the thiamine status of this herd. Blood samples were taken (sampling 1) for measurement of concentrations of Cu, Zn, Fe and thiamine from approximately 70% of the animals in both pens on February 6. Samples of all feed ingredients, drinking water and mineral mix (Table 2) were taken for analysis of Cu, Zn, Fe, Manganese (Mn), molybdenum (Mo), selenium (Se) and total sulfur (S). Based on analysis of trace minerals (Tables 1 and 2), total intake of Cu by the animals was found to inadequate (Table 3). A custom-made micromix (Table 4) to supplement the existing mineral supplement was advocated on April 2, 1987. Feeding of this newly mixed supplement commenced on April 5, 1987. Three weeks later (on April 25), samples of feed, water and blood (sampling 2) were collected as outlined previously, from approximately 34% and 27% of the animals in north and south pens respectively.

Table 3. Copper, Zn, Fe, Mo and S intake by cattle prior to and after micropremix supplementation.

	Pen	Mineral intake				
		Cu	Zn	Fe	Mo	S
		(mg kg <sup>-1</sup> DM)				
Prior to Supplementation <sup>a</sup>	North & South	7.1	31	257	0.13	0.38
After Supplementation <sup>b</sup>	North	57.5	126	268	0.38	0.38
	South	37.0	86	245	0.38	0.38

<sup>a</sup> Based on average daily intakes of 2.72 kg forage, 4.1 kg barley and 0.9 kg conola meal (see Table 1 for analysis) and 70 g min. mix 1 [prior to supplementation (see Table 2)]. Daily water intake was assumed to be 18 L per animal.

<sup>b</sup> Feed intake was similar to above but intake of custom made micropremix (see Table 4) varied between the animals in the two pens; 133 g by steers in the north pen compared to 78.5 g by those in the south pen.

Table 4. Manufacturer's guaranteed and laboratory analysis of custom made micropremix used to supplement the existing mineral mix after diagnosis of trace mineral deficiency.

Mineral	Guaranteed analysis by manufacturer <sup>a</sup> (mg kg <sup>-1</sup> )	Laboratory analysis (mg kg <sup>-1</sup> )	Daily intake of mineral/animal <sup>b</sup> (mg) after micropremix supplementation	
			North Pen	South Pen
Cu	50,000	69,658	359	212
Zn	140,000	131,878	700	413
Fe <sup>c</sup>	d	6,967	429	254
Mo <sup>c</sup>	d	65	0.36	0.21
Mn <sup>c</sup>	d	519	15.0	12.0
Se	400	480	2.48	1.60
S <sup>c</sup>	d	3.18%	0.61g	0.36g

<sup>a</sup> mg kg<sup>-1</sup> DM. The micropremix also contained 3x10<sup>7</sup> IU kg<sup>-1</sup> of vitamin A, 4x10<sup>6</sup> IU kg<sup>-1</sup> of vitamin D<sub>3</sub>, and 10,000 IU kg<sup>-1</sup> vitamin E.

<sup>b</sup> Mineral supplementation from April 5 consisted of 1.6 kg micropremix, 25 kg min. mix, 8.2 kg calcium carbonate, 6.8 kg of blue salt (sodium chloride, iodine, cobalt). Daily intakes of minerals have been estimated from average intakes of 133 g and 78.5 g per day by steers in north and south pen respectively.

<sup>c</sup> these minerals were not recommended but appeared in the micropremix as contaminants. <sup>d</sup> not available.

**Analytical Techniques:** Concentrations of Cu, Zn and Fe in plasma were determined after dilution with deionized water (v/v; 1:3) followed by precipitation with 1 volume of 20% trichloroacetic acid (TCA) and centrifugation. The plasma supernatants so obtained and farm water samples were measured directly on an atomic absorption spectrophotometer (AAS) (Perkin Elmer, model 5000). Concentrations of Cu, Zn, Fe, Mn, Mo, and Se from samples of forage, barley, canola meal and mineral supplements were also measured on AAS after digestion with a nitric: perchloric acid (3.5:1 v/v) mixture.

Levels of S in forage, barley, canola meal and mineral supplements were analyzed by induction coupled plasma emission spectroscopy (ARL Model 3410) after digestion in nitric and perchloric acids as described by Blanchard et al. (11).

Determinations of blood thiamine were carried out by the method of Olkowski et al. (unpublished), a modification of the method of Sarett and Cheldelin (see appendix).

**Statistics:** Student's t-test (13) was used to determine pre- and postsupplementation of micropremix (main effect) on concentrations of Cu, Zn, Fe in plasma and thiamine in blood. The secondary effects between breeds (Simmental-crosses and others), and pens (north and south pen) were tested using analysis of variance (ANOVA) and Duncan's multiple range test (14). Differences of  $p < 0.05$  are reported as statistically significant.

## RESULTS

**Composition of diets and trace mineral intakes:** Cu and Zn had been omitted inadvertently by the manufacturer from the mineral mix (Table 2). Based on feed analysis (Table 1) and a daily consumption of 2.7 kg forage, 4.1 kg barley, 0.9 kg canola meal, 70 g of mineral supplement (Table 2), and a water intake of 18 L (2), we calculated the daily intake of Cu, Zn, Fe and Mo by each steer in the north pen to be 50, 169, 1800 and 0.92 mg respectively. Intakes of S via feed and water were 18.6 and 7.9 g respectively. Values for steers in the south pen were approximately 10% less than values for steers in the north pen. Based on 90% feed DM, we calculated the resulting concentrations of Cu, Zn, Fe, Mo to be 7.1, 31, 257 and 0.13 mg/kg of feed DM (Table 3). Similarly, intake of S was 0.38% feed DM (Table 3).

Supplementation with a Micropremix (initiated on April 5) increased the daily intakes of Cu and Zn several fold (Table 4). This was most apparent for animals in the north pen because of their higher consumption of this supplement. The intakes of other microminerals also increased but only slightly, whereas the intakes of macrominerals including S did not change to any great extent following supplementation of the micropremix (Table 3).

**Growth and general appearance of animals:** Examination of the herd on the date of the first blood sampling (February 6) showed evidence of Cu deficiency including lack of desire to feed, unthriftiness, and loss of condition with typical dull, rough hair coat and loss of pigment in hair (Figure 1). Three weeks of

supplementation with the micropremix was sufficient to markedly improve the overall condition of the animals (Figure 2). All animals showed improved body condition, weight gain, and return of normal coat luster and color of hair.

**Copper status:** Copper status of calves at the first sampling, as assessed by concentration of plasma Cu, was low (Table 5). Concentration of Cu in plasma of less than 0.3 mg/L has been associated with Cu deficiency in cattle (13). Thus, approximately 45% of the animals were Cu deficient (Figure 3). Animals in the north pen appeared to be the most severely affected. Simmental crosses had lower concentration of plasma Cu than the other breeds in the herd. As expected, supplementation of the micropremix resulted in a significant increase ( $p < 0.01$ ) in concentration of Cu in plasma of all steers except the Simmental crosses in the south pen. The latter result may not represent true Cu status of this group since only two Simmental crosses from the south pen were sampled at this time. At sampling 2, following supplementation with the micropremix, only 3% of the animals had Cu levels in plasma less than 0.3 mg/L. Concentration of Cu in plasma of the steer which developed PEM was 0.35 mg/L at sampling 1 but declined to 0.33 on March 3. After three weeks of supplementation with the micropremix, the concentration of Cu in plasma had increased to 0.55 mg/L.

**Iron status:** The levels of consumption of Fe before and after supplementation with the micropremix were similar (Table 3).



Fig.1 The appearance of a steer prior to trace mineral supplementation. Note the depressed state of the animal and its dull, rough and depigmented coat.



Fig.2 Appearance of the same animal after 3 weeks of mineral supplementation. Note marked improvement in body condition, coat texture and coat color.

Table 5. Plasma Cu concentration<sup>a</sup> of the animals prior to and after micropremix supplementation.

	Plasma Cu concentration (mg L <sup>-1</sup> )				
	<u>Both Pens</u>	<u>North Pen</u>		<u>South Pen</u>	
			Simmental Crosses	Others	Simmental Crosses
<b>Prior to Supplementation<sup>b</sup></b>	0.42 <sup>d</sup> ± 0.16 (n=77)	0.34 <sup>f</sup> ± 0.11 (n=17)	0.38 <sup>f</sup> ± 0.14 (n=21)	0.42 <sup>fg</sup> ± 0.16 (n=7)	0.48 <sup>g</sup> ± 0.16 (n=32)
<b>After Supplementation<sup>c</sup></b>	0.68 <sup>e</sup> ± 0.15 (n=32)	0.72 <sup>h</sup> ± 0.14 (n=8)	0.70 <sup>h</sup> ± 0.19 (n=10)	0.45 <sup>fg</sup> ± 0.21 (n=2)	0.67 <sup>h</sup> ± 0.10 (n=12)

<sup>a</sup> Results are expressed as mean ± SD

<sup>b</sup> Prior to supplementation refers to sampling 1 on February 6, 1987.

<sup>c</sup> After supplementation refers to sampling 2 on April 25, 1987 (taken 3 weeks after commencement of micropremix supplementation).

<sup>de</sup> Values with different superscripts differ significantly [(P<0.01) Student T test]

<sup>fgh</sup> Values with different superscripts differ significantly [(P<0.05) Duncan's multiple Range Test]

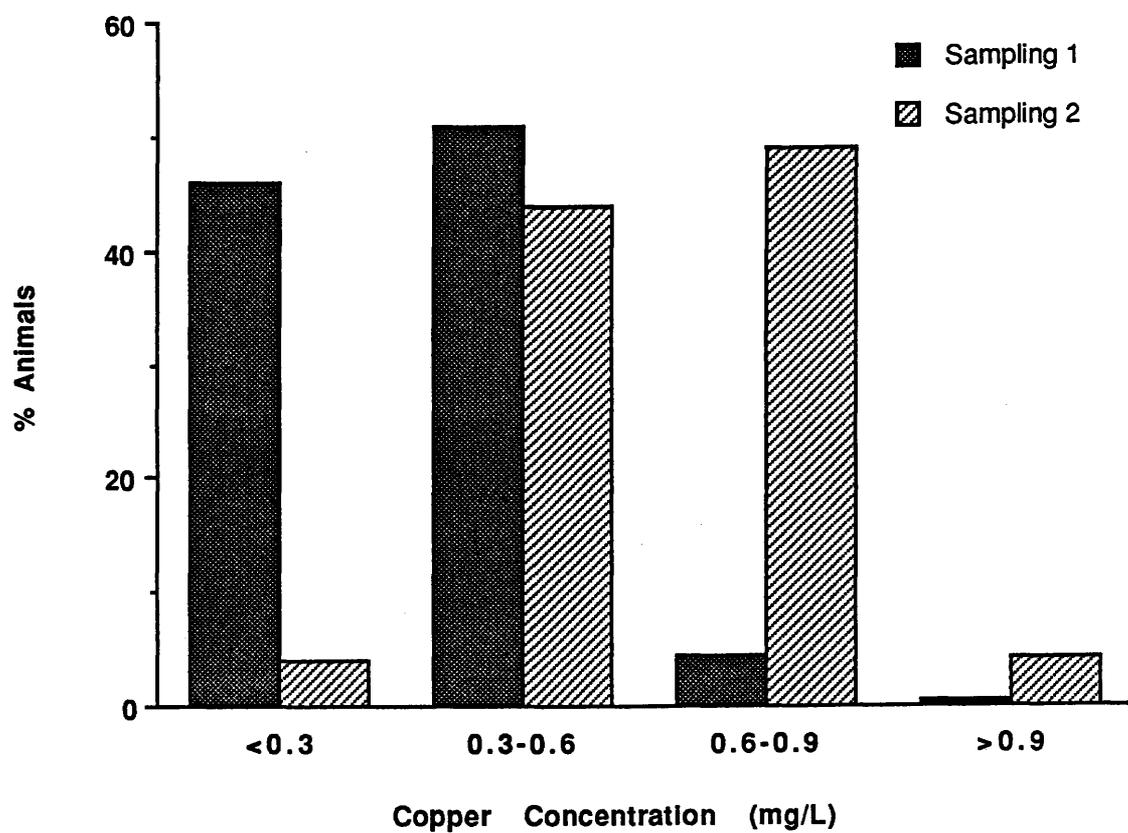


Fig. 3. A comparison of plasma copper levels in cattle prior to (sampling 1) and after (sampling 2) micropemix supplementation.

The concentration of Fe (mean  $\pm$  SD) in plasma of cattle after premix supplementation at sampling 2 were significantly lower ( $2.15 \pm 0.42$  mg/L) ( $p < 0.05$ ) compared to the values at sampling 1 ( $2.73 \pm 0.47$  mg/L).

**Zinc status:** Consumption of Zn increased three-to-four fold after supplementation with the premix, but the mean plasma Zn concentration of all animals increased only slightly ( $p > 0.05$ );  $1.44 \pm 0.26$  mg/L at sampling 2 compared with  $2.37 \pm 25$  mg/L at sampling 1.

**Thiamine status:** Mean ( $\pm$  SD) blood thiamine concentration at sampling 1 (prior to micropremix supplementation) was  $49.2 \pm 14.9$   $\mu$ g/L. Approximately 30% of animals in the herd at this time had concentrations of thiamine less than 40  $\mu$ g/L in blood (Figure 4). Levels in Simmental cattle and other breeds were similar. After micropremix supplementation, concentration of thiamine in blood increased significantly ( $p < 0.05$ ) in all cattle to  $128.2 \pm 41.7$   $\mu$ g/L (Figure 4).

Only the steer which developed PEM received thiamine supplementation. After a single injection of thiamine, the concentration of thiamine in blood of this animal increased to 320.3  $\mu$ g/L, but it declined to 36.1  $\mu$ g/L four weeks later. No supplementation of thiamine was given after February 10, but three weeks of supplementation with the micropremix alone increased the concentration of thiamine in blood to 96.9  $\mu$ g/L in this steer.

## DISCUSSION

It is evident from the investigation reported here that a combination of excessive intake of S, and low dietary intake of trace minerals, especially Cu, is

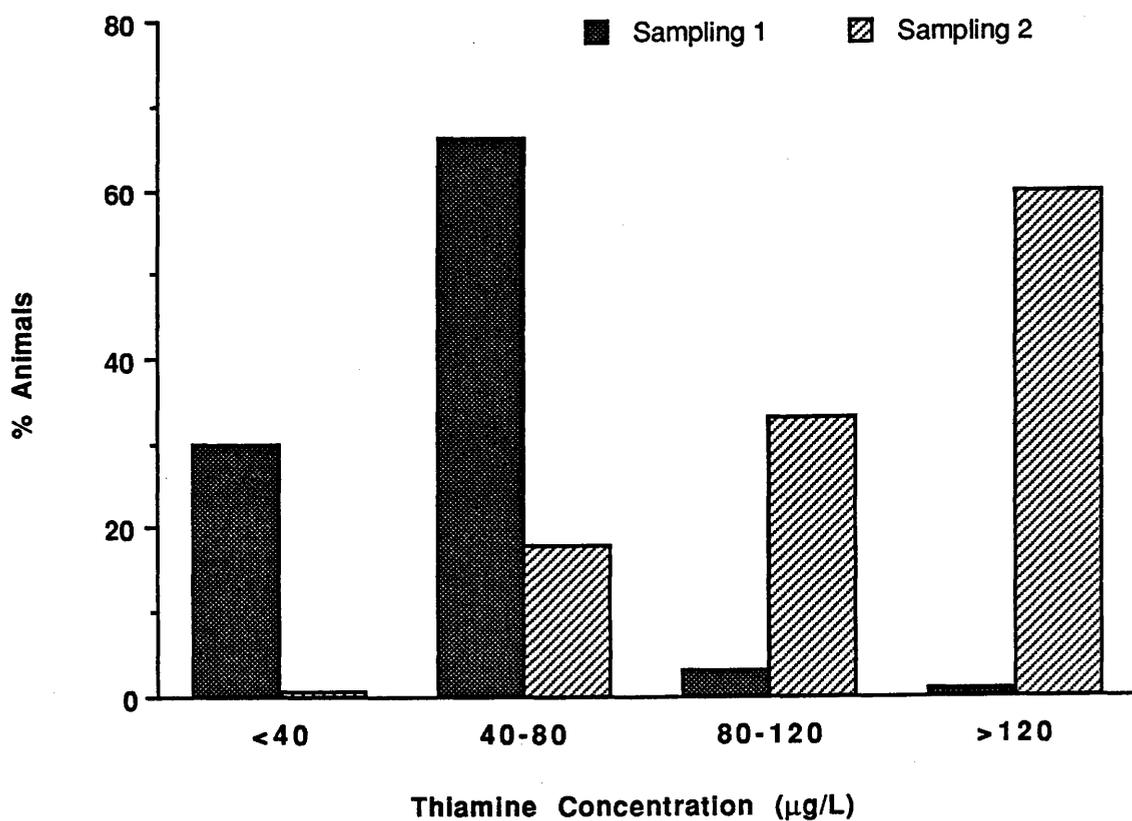


Fig. 4. A comparison of blood thiamine concentrations in cattle prior to (sampling 1) and after (sampling 2) micropemix supplementation.

detrimental to the thiamine status in cattle. Based on analysis of thiamine in blood of cattle in this study, it might be expected that on the Prairies there are many cattle which suffer from subclinical deficiency of thiamine.

Supplementation of trace minerals alone resulted in a significant improvement in both the thiamine and Cu status of cattle exposed to an above-normal level of S. Although PEM has been associated with a high concentration of S in the diet (6) and drinking water (8), to our knowledge this is the first time a trace mineral-responsive thiamine deficiency has been documented. We believe that the events leading to a deficiency of thiamine as reported here are due to a series of complex interactions of microminerals (Cu, Zn, Fe, Mo), macrominerals (S) and thiamine.

Interactions among Cu, Zn, Fe, Mo, and S are widely recognized (15). The bioavailable fraction of each of these elements can be regarded to be "in balance" with that of the other four, and hence the changes in concentration of one would distort the metabolism of the others (15). In this context it is notable that concentration of Zn in plasma was lower at sampling 2 in spite of a three- to fourfold increase in intake of dietary Zn after supplementation with the micropremix. This may have resulted from a decreased absorption of dietary Zn because the concentration of Cu in the diet of animals in the two pens increased from 7 to 37.0 (south pen) and 57.5 (north pen) mg/kg during this period (Table 3). Similarly, the concentration of Fe in plasma of animals was also significantly lower at sampling 2. This is not surprising since excess dietary Cu, relative to Fe,

is known to reduce the Fe status in cattle, and vice versa (16). It has been shown that 250 mg/kg DM of Fe in the diet can induce Cu deficiency in cattle (17) and accumulation of Fe has been noted in such animals (18). Copper deficiency produces anemia and a characteristic response to anemia is an enhanced efficiency in Fe absorption (19). Thus a relatively higher concentration of Fe in plasma observed at sampling 1 (prior to supplementation with the micropremix) was probably due to an enhanced absorption of Fe by Cu-deficient animals from the diet which contained low Cu, moderately high Fe, and marginal Zn.

In ruminants, both inorganic and organic S compounds are reduced to sulfides by rumen bacteria. Sulfides bind with a variety of divalent cations to form cationic sulfides. Affinity of S for Cu is higher than other cations and the solubility product of Cu sulfide is extremely low ( $3.48 \times 10^{-38}$ ). In addition, thiomolybdates (TM) which form in the rumen at moderate to high intakes of S and Mo, form unavailable Cu-TM complexes which exert effects both in the gut and systemically (7), further reducing the availability of Cu to the animal. Breed differences in Cu metabolism are recognized and Simmental cattle appear to be more susceptible to Cu deficiency than other breeds (7). This is supported by the present study in that the concentrations of Cu in plasma of the Simmental crosses were lower than those in the other breeds (Table 5). Hypocupremia was evident at sampling 1 (Table 5) with approximately 45% of the animals deficient in Cu (Figure 3). Depigmentation and rough hair coat observed at this time (Figure 1) are typical manifestations of Cu deficiency (20). Reversal of these signs within

three weeks of supplementation with the micropremix suggest synthesis of copper-dependent enzymes such as polyphenyloxidases which are involved in pigmentation and keratinization of hair. It was not surprising to find an improvement in growth and body condition after supplementation with micropremix. Significant increases in weight gains, have been reported in previously deficient cattle when supplemented with Cu (21) and thiamine (22). Therefore it is reasonable to believe that the improved animal performance observed in the present study was due to a combined improvement in Cu and thiamine status in these animals.

Recent evidence that excess dietary S also exerts an effect on availability of thiamine in ruminants (10) suggests the possible occurrence of a complex interaction of thiamine - S - divalent cations in the rumen. If this is true, a low concentration of divalent cations in the diet would indirectly result in a relatively higher concentration of "free" sulfide in the rumen and this may create a thiamine deficiency. We believe that this may have been the reason for the depleted levels of thiamine in blood (sampling 1) observed in the present study prior to supplementation with the micropremix. Conversely, if the diet is supplemented with normal or a slight excess of cations, especially with Cu, it would result in formation of cationic sulfides. This would lower the concentration of free sulfide in the rumen (23), and thereby alleviate the effects of S-induced thiamine deficiency. This is probably the most likely explanation for the significant increase in levels of thiamine observed in blood of animals in the present study

after supplementation with the micropremix. The response was immediate, since an improvement in thiamine status was observed within three weeks of supplementation. This improvement in thiamine status occurred in spite of no change in basal diet, drinking water supply or any supplementation with thiamine.

Clinical signs of thiamine deficiency have been divided broadly into those attributable to general metabolic disorders, which may be observed at an early stage, and those related to disorders of the central nervous system (24). It is interesting to note that the clinical signs listed as metabolic disorders of thiamine deficiency such as transient scouring, reduced growth rate, anorexia, general unthriftiness and reproductive disorders are also common signs of chronic Cu deficiency. We believe that, under field conditions, deficiencies of both Cu and thiamine may exist concurrently in cattle having access to excessive levels of S. In instances in which high S-related PEM is diagnosed, conventional thiamine therapy (25) is beneficial and clinical recovery occurs within one to three days. However, as observed in the present study, the elevation of concentration of thiamine after IV thiamine therapy is only temporary. As long-term therapy, we recommend that herds exposed to high intakes of S also be supplemented with Cu up to 50 mg/kg feed DM. This could be expected to alleviate deficiencies of both Cu and thiamine.

In a previous experiment, two of eight cattle fed 0.5% S and 10 mg Cu/kg DM in the diet, developed PEM within six weeks. Concentrations of thiamine in

blood of affected animals were 14.7 and 23.5 µg/L (Gooneratne et al. unpublished observations). Based on our experience, we believe that a concentration of less than 40 µg/L of thiamine in blood could be considered as marginal. Thus, approximately 30% of the animals in the present study were deficient at sampling

1. Depression of concentration of thiamine in blood has been reported previously in cattle fed diets adequate in Cu but supplemented with 0.72% sulfate (10). Since ruminants do not require thiamine in their diet, one or several of the following mechanisms may have been responsible for the lower levels of thiamine in plasma as observed in the present study: inadequate microbial synthesis of thiamine, impaired absorption and utilization of thiamine, presence of thiamine antimetabolites, lack of apoenzyme, increased metabolic demand for thiamine, or increased rate of thiamine excretion (3). It is not clear which of these pathways are involved in the induction of S-related thiamine deficiency. However, there is some evidence that diets high in S decrease the amount of thiamine entering the duodenum, probably due to a reduced synthesis of thiamine (10), and increased excretion of thiamine (Olkowski et al. unpublished observations). High intakes of S decrease ruminal, duodenal and fecal pH (26). Ruminal acidosis appears to establish conditions conducive to development of PEM (27), such as enhanced production of the thiamine-destroying enzyme, thiaminase (28), and hence a critical concentration of extracellular thiamine in rumen fluid may be an important factor in the control of thiaminase production.

## Acknowledgments

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**ASSOCIATION OF SULFATE - WATER AND BLOOD THIAMINE  
CONCENTRATION IN BEEF CATTLE: FIELD STUDIES**

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**ABSTRACT**

A population study was undertaken to survey the distribution of sulfate levels in water and thiamine status of beef cattle on Saskatchewan farms. A total of 50 farms took part in this study. The sampled animals represented all major breeds raised in Saskatchewan. The sulfate content in drinking water varied greatly across the province ranging from 70 to 3200 ppm. Approximately 43% of the farms from southern and central parts of the province had water with sulfate concentration exceeding 1000 ppm. The concentration of blood thiamine was (mean  $\pm$  SD)  $24.9 \pm 10.1 \mu\text{g L}^{-1}$ . Subsequently, a comparative study was undertaken to examine the relationship between high sulfate in the drinking water and blood thiamine concentration in feedlot cattle. The farms with high (>1000 ppm) and low (<200) levels of sulfate in the water were used for the comparative study. Blood thiamine concentrations differed ( $P < 0.0001$ ) between beef cattle drinking low sulfate-water and those drinking high sulfate-water and were  $47.3 \pm 9.8$  and  $37.9 \pm 12.4 \mu\text{g L}^{-1}$  respectively. It was concluded from this study that a high concentration of sulfate in the drinking water may have a detrimental effect on blood thiamine status. A subpopulation of beef cattle in Saskatchewan may be marginally deficient or deficient in thiamine.

## INTRODUCTION

The sulfate content of water on many farms throughout the Canadian Prairies is considered to be excessive for animals (Boila 1988; Patience et al. 1989). A recent survey of hog farms across the province of Saskatchewan revealed that 25 % of farms use water containing more than 1000 ppm of sulfate, i.e. exceeding the recommended maximum (Patience et al. 1989). Another survey showed that 18 % of dairy farms use water with sulfate concentration greater than 1000 ppm (McLean and Christensen, personal communication). The recommended dietary sulfur (S) level (organic and inorganic forms) for beef cattle is 0.08 - 0.15% (National Research Council 1984). Dietary S above 0.3 - 0.4% may cause toxic effects (Kandyliis 1984). Therefore, large numbers of animals raised on the prairies may be exposed to drinking water containing levels of sulfates which can be considered to be potentially harmful to the animal.

Consumption of water containing a high concentration of sulfate has adverse effects in cattle. Weeth and Hunter (1971) observed that 3500 ppm sulfate in drinking water reduced water and feed consumption and caused loss of body weight. Water intake was not affected by sulfate added to water at a rate of 2800 ppm, but this concentration resulted in decreased feed intake and lower weight gains (Weeth and Capps 1972). Cows and calves had lower body weight and there was increased mortality of calves during the postnatal period when animals were drinking water containing 1500 ppm of sulfate (Smart et al. 1986). Palatability of water is also a problem. Cattle were able to discriminate against

water containing 1450 ppm sulfate and reject water with 2150 ppm if more palatable water was given as an alternative, but they would drink sulfate-water which is injurious if no other water was available (Weeth and Capps 1972).

The detrimental effects of high dietary S on thiamine metabolism may be of particular importance in ruminants. Sulfate is reduced to sulfite and sulfide by the rumen microorganisms (Lewis 1956). Very little sulfate is absorbed whereas sulfide is absorbed rapidly (Bray 1969). Hence, the recycling of sulfate to the rumen (Kennedy et al. 1975) must go through the cycles of reduction to sulfite and sulfide by rumen bacteria (Lewis 1956). Sulfite ions in-vitro cleave the thiamine molecule (Leichter and Joslyn 1969).

Thiamine deficiency can occur in cattle fed high dietary levels of sulfate (Goetsch and Owens 1987). A recent field study suggested that there is an association between high sulfate in drinking water and thiamine deficiency in cattle (Gooneratne et al. 1989). High sulfate intake has been incriminated in the induction of polioencephalomalacia (PEM) (Raisbeck 1982; Sadler et al. 1983; Harries 1987; Gooneratne et al. 1989), a central nervous system (CNS) disorder that is commonly believed to be due to thiamine deficiency (Pill 1967; Dickie et al. 1979).

A population study was undertaken to survey the distribution of sulfate levels in water and thiamine status of beef cattle on Saskatchewan farms. Following this, a comparative study was undertaken to establish whether there is a

relationship between high sulfate in the drinking water and blood thiamine concentration in feedlot cattle.

## **MATERIALS AND METHODS**

**Population Study.** A total of 50 beef farms representing large scale feedlot operations as well as small producers from major geographic regions of Saskatchewan (south, central, north) were used. Blood samples were taken from 221 animals ranging from 3 to 14 mo of age. The sampled animals represented all major breeds raised in Saskatchewan. An attempt was made to assure random order of selection. However, as this study was based on voluntary participation, the randomization was limited to those producers who responded to our request and agreed to lend their animals for sampling. The animals on each farm were sampled randomly.

**Comparative study.** The selection of farms was based on information gathered during the population study. The farms used in this study provided animals exposed exclusively to either high (>1000 ppm) or low (<200 ppm) levels of sulfate in the water. A total of 18 farms were used in this study. Blood samples were obtained from 400 feedlot beef cattle 8 to 16 mo of age. One hundred ninety nine animals were sampled from ten farms with a low level of sulfate and 201 animals were sampled from eight farms with high sulfate in the drinking water.

**Sample Collection.** Water samples were collected into plastic vials. Blood samples were obtained from the jugular vein into glass vacutainers containing

heparin (Becton Dickinson, Rutherford, NJ). Blood samples were transferred to plastic tubes. Water and blood were stored at -20°C until analyses.

### **Analytical Procedures**

**Water sulfate.** Water sulfate analysis was performed as described by Sörbo (1987).

**Blood thiamine.** Blood thiamine was measured as described by Olkowski and Gooneratne (1991, see appendix). The method is a modification of the assay of Baker and Frank (1968). Briefly, blood was hemolyzed by freezing at -20°C. Thiamine was extracted by mild acid digestion (trans-aconitate pH 4.0) at 115°C for 20 min. A microbiological assay utilizing *Ochromonas danica* was performed on a 96 well microplate and growth optical density was read on a microplate reader (Vmax, Molecular Devices Corp. Palo Alto, CA) at 650 nm.

**Statistics.** Statistical analyses were carried out using General Linear Models from a microcomputer package Number Cruncher Statistical System (Hintze 1987). Differences among the groups were analyzed using one way analysis of variance (Snedecor and Cochran 1989). Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

## **RESULTS**

**Population Study.** The sulfate content in drinking water for cattle varied greatly across the province ranging from 70 to 3200 ppm. Approximately 43% of the farms from southern and central parts of the province had water with sulfate concentration exceeding 1000 ppm (Table 1).

Table 1. Sulfate content in drinking water (wells or dugouts) on Saskatchewan farms

Region	Farms sampled	Water Sulfate (ppm)		
		< 300	300-1000	>1000
		Distribution (%)		
Southern	14	21	36	43
Central	19	26	32	42
Northern	17	59	41	0
Total farms	50	36	36	28

The mean  $\pm$  SD concentration of blood thiamine was  $24.85 \pm 10.1 \mu\text{g L}^{-1}$ . The distribution of Saskatchewan cattle blood thiamine is shown in Fig.1. Approximately 30% of the animals had a blood thiamine concentration of less than  $20 \mu\text{g L}^{-1}$ . There were regional differences ( $P < 0.013$ ) in blood thiamine concentrations with means  $\pm$  SD being:  $27.0 \pm 10.8$ ,  $22.5 \pm 7.4$ , and  $24.5 \pm 12.1 \mu\text{g L}^{-1}$  for cattle from the northern, central and southern region, respectively.

**Comparative study.** Beef cattle drinking water containing a high level of sulfate had lower ( $P < 0.0001$ ) blood thiamine concentrations in comparison to cattle drinking low sulfate water (Table 2). The distribution of blood thiamine in relationship to the sulfate level in drinking water is shown in Fig.2. Approximately 20% of sampled cattle consuming high sulfate water had blood thiamine concentrations below  $30 \mu\text{g L}^{-1}$ . In contrast, only 2.5% of animals from the low sulfate farms fell into this category.

## DISCUSSION

A level of 2500 ppm sulfate in drinking water for cattle was proposed by Digesti and Weeth (1976) as a safe tolerance concentration. However, in view of current knowledge on S toxicity in cattle, this level appears to be too high. Water intake by cattle depends on ambient temperature and can vary from  $3 \text{ L kg}^{-1} \text{ DM}$  at  $5^\circ \text{C}$  up to  $8 \text{ L kg}^{-1} \text{ DM}$  at  $32^\circ \text{C}$  (National Research Council 1984).

Hence, the intake of 1000 ppm sulfate-water at a rate  $6 \text{ L kg}^{-1} \text{ DM}$  can increase the total dietary S by approximately  $2 \text{ g kg}^{-1} \text{ DM}$  or 0.2%. Dietary S above 0.3 - 0.4% may cause toxic effects (Kandylis 1984).

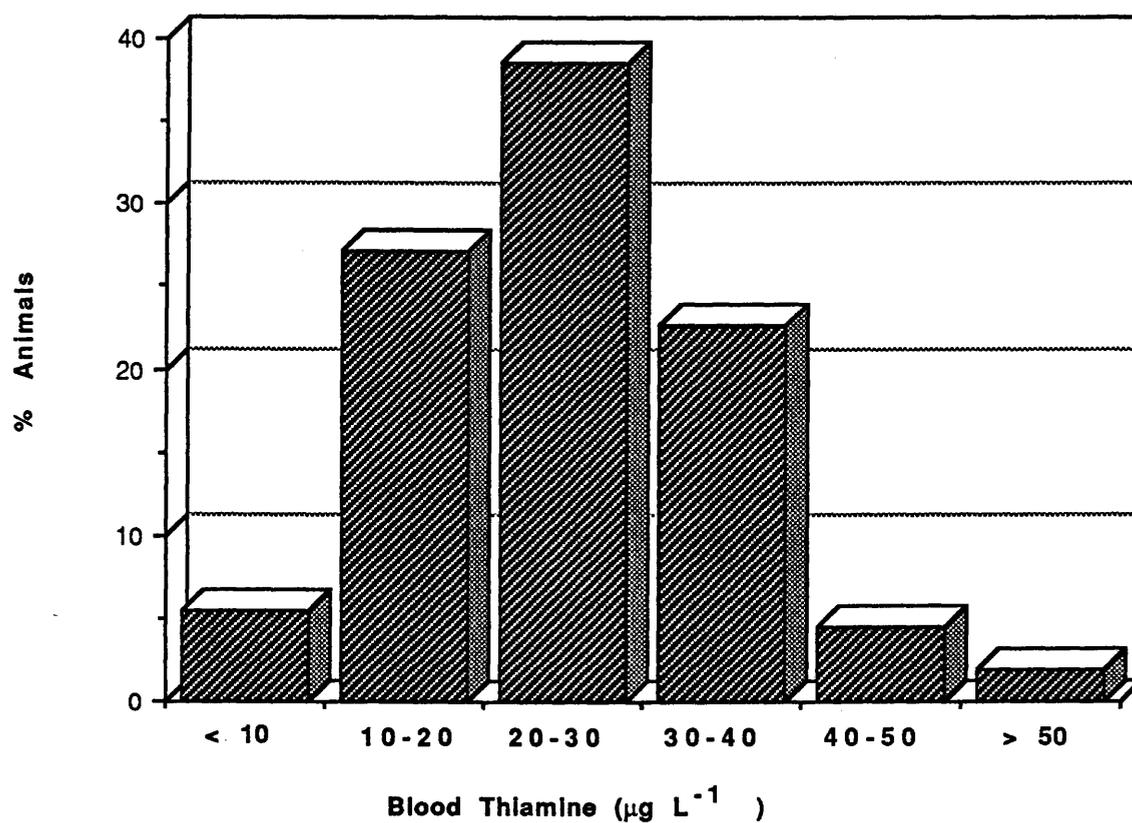


Fig. 1. Population distribution of blood thiamine concentration in beef cattle in Saskatchewan. Total of 221 animals were sampled.

Table 2. Blood thiamine concentration in beef cattle receiving low (<200 ppm) and high (>1000 ppm) sulfate in the drinking water

Sulfate level	(n)	Blood Thiamine ( $\mu\text{g L}^{-1}$ )	Range
Low (<200 ppm)	199	47.3b	21.0 - 75.6
High (>1000 ppm)	201	37.9a	2.5 - 72.1

SEM 0.9

a,b indicate means differed ( $P < 0.0001$ )

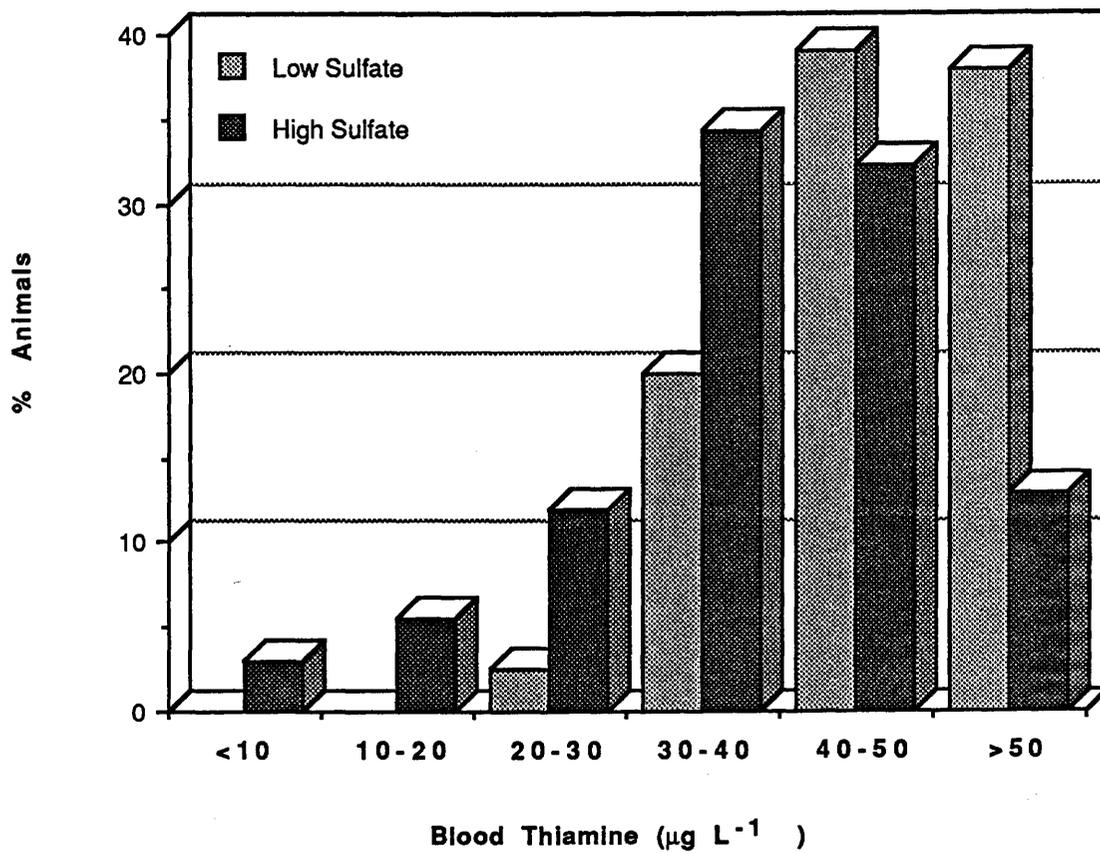


Fig. 2. The distribution of blood thiamine in relation to sulfate level in drinking water. One hundred ninety nine cattle drinking low sulfate-water (<200 ppm), and 200 cattle drinking high sulfate-water (>1000 ppm) were sampled.

Thus, even by very conservative estimates, ignoring the possibility of high to excessive levels of S in feed (Boila et al. 1987), water alone at 1000 ppm sulfate concentration can increase dietary S to a toxic level. In fact, dietary S at a level 0.5% induced PEM in cattle within 6 wks (Gooneratne et al. 1989).

The present study has shown that cattle exposed to a high (>1000 ppm) level of sulfate in water had blood thiamine concentrations lower ( $P < 0.0001$ ) than those drinking low sulfate (<200 ppm) water. The significance of this finding is not known. However, there is a possibility that a sub-population of cattle raised on farms using high sulfate-water may be deficient in thiamine. Indeed, from this study it appears that sulfates in the water may be associated with lower blood thiamine status in cattle. Approximately 20% of the cattle drinking high sulfate water had deficient blood thiamine levels. However, since several animals drinking low sulfate water also had low blood thiamine concentrations, it is likely that S alone is not the sole factor causing decreased blood thiamine.

Cattle from the northern part of the province had higher blood thiamine concentration than those from the central or southern regions. This is consistent with the overall findings since the highest concentrations of sulfate in the water were found in central and southern Saskatchewan. Considering that the feeds grown in northern Saskatchewan are of poorer quality (Racz, personal communication)<sup>a</sup>, hypothetically, a higher blood thiamine concentration in animals from the north may be associated with better quality of water.

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<sup>a</sup> On the basis of the data from annual surveys of feeds grown in Saskatchewan. Feed Testing Lab., University of Saskatchewan.

The results from this study indicate that approximately 30 - 40 % of animals tested during the population study may have had an inadequate blood thiamine concentration. Such a high percentage appears to be in conflict with data obtained from the comparative study. However, these results should be interpreted with caution. It is notable that the population study was conducted during hot summer months whereas the comparative study was conducted in late fall. The higher rate of water consumption during the summer may have elevated the S intake by relatively more animals. It appears that a marginal thiamine deficiency may be more prevalent.

There is insufficient data to define what constitutes a normal blood thiamine level in ruminants. Values reported earlier were largely inconsistent and may not be reliable because of non-specific analytical methods (Loew and Dunlop 1972, Rammell and Hill 1986). The recently published work of Hill et al. (1988) proposed blood thiamine reference values of 25.3 - 62.4  $\mu\text{g L}^{-1}$  for normal sheep and cattle on pasture. However, these reference values may be too low for feedlot animals since Loew (1975) has suggested that thiamine deficiency can be a problem in cattle fed a high carbohydrate diet due to a higher metabolic demand for thiamine. Grigat and Mathison (1982) concluded that decreased production in cattle fed all-concentrate diets is possibly due to marginal thiamine inadequacies.

Based on our previous laboratory and field experience (Gooneratne et al. 1989; Olkowski et al. unpublished observations) and the information gathered in the

course of this study, some general guidelines can be proposed. Gooneratne et al. (1989) reported blood thiamine concentrations of 14.7 and 23.5  $\mu\text{g L}^{-1}$  in two heifers fed high S which developed PEM. A feedlot (using 3200 ppm sulfate water) that took part in our study experienced an outbreak of PEM in the year preceding this study<sup>b</sup>. Blood thiamine concentration was not measured during the outbreak, but the measurements obtained from this farm in the present study showed a blood thiamine concentration of (mean  $\pm$  SD)  $16.9 \pm 10.4 \mu\text{g L}^{-1}$ . In our view, a blood thiamine concentration of 25 - 30  $\mu\text{g L}^{-1}$  can be considered marginally deficient, and below 25  $\mu\text{g L}^{-1}$  deficient, in feedlot cattle.

The pathogenesis of thiamine deficiency is not well understood. The condition can occur as a general metabolic disorder or a CNS disorder (Rammell and Hill 1986). The CNS disorder (PEM) appears to be an effect of prolonged and more severe thiamine inadequacy (Thornber et al. 1979). It is not clear whether or not thiamine deficiency is the factor which causes the disease or if it is only a predisposing factor, but there is little doubt that thiamine inadequacy is implicated in this condition (Blood and Radostits 1989). Although not all animals with low blood thiamine concentration will develop clinical signs of PEM, they may be at higher risk particularly when exposed to high dietary S. The study by Raisbeck (1982) indicated that cattle fed excess S were 46 times more likely to develop PEM than those fed normal S diets. A number of recent reports have shown that PEM can be induced upon feeding excess dietary S (Raisbeck 1982;

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<sup>b</sup>Diagnosed by Dr. A.Y. Kernaleguen, DVM, Stoughton Veterinary Clinic, Stoughton, Saskatchewan.

Sadler et al. 1983; Rousseaux et al. 1991). Polioencephalomalacia was also observed during feeding trials where excess dietary S was fed to cattle (Khan et al. 1987; Gibson et al. 1988). An association between PEM and a high level of sulfate in drinking water was also shown (Harries 1987; Gooneratne et al. 1989). Polioencephalomalacia is the most prominent and easily observable thiamine related condition, but it appears to be diagnosed only sporadically. However, the disease may occur more often in a subclinical form. In experimentally induced cases not all animals with morphologic PEM lesions showed clinical signs (Rousseaux et al. 1991), attesting to the problem of recognizing thiamine deficiency in ruminants.

General metabolic disorders associated with thiamine deficiency, albeit not as drastic as PEM, may be of more economical importance. Recognition of marginal thiamine deficiency from a nutritional standpoint may be very difficult. In practice, the diagnosis of thiamine deficiency is not made unless obvious clinical (most often PEM) signs occur in the herd. However, it is natural that before the obvious signs appear there is probably a quite long latent period, which no doubt causes metabolic distress to the animal. Thus, subclinical thiamine deficiency may go undetected for long periods of time. In addition, the signs of marginal thiamine deficiency may not always be easily recognized as such. Even in more advanced general disorders cases clinical signs such as scouring, reduced growth rate, weight loss, anorexia, unthriftiness, and agalactia observed in thiaminase-related thiamine deficiency (McDonald 1982, Thomas 1986) are not specific and

may be attributed to a variety of causes. Hence, the potential source of the problem may be easily overlooked by producers.

According to the present standards ruminants are not considered to require thiamine supplementation (National Research Council 1984). However, both subclinical and acute thiamine deficiency do occur in ruminants (Rammell and Hill 1986). Brent and Bartley (1984) postulated that the assumption of self-sufficiency in ruminant species for B vitamins should be reexamined. Supporting their comments, recent research indicates that subclinical thiamine deficiency in ruminants may be more common if animals are exposed to excess dietary S (Goetsch and Owens 1987; Gooneratne et al. 1989; Rousseaux et al. 1991).

Notably, the findings on S toxicity in past research were not consistent (for review see Kandylis 1984). Some authors fed S levels as high as 1.72% in feed (Chalupa et al. 1971) or 7000 ppm sodium sulfate in water (Embry et al. 1959) with no observable deleterious effects, whereas others observed reduced performance when S levels were substantially lower (Weeth and Hunter 1971; Weeth and Capps 1972; Bouchard and Conrad 1973). Interestingly, a decrease in tolerance of cattle for S and drastic change in clinical picture appears to be a quite recent phenomenon. Raisbeck (1982) was the first to observe an association between high dietary S and PEM in cattle. Since then more evidence (cited above) from both veterinary and nutritional research, attests that this problem is increasingly recognized. The reason for this is not known. However, a plausible hypothesis is that the genetic selection for better performance could produce

animals so refined that, under the stress of modern production practices they would respond with serious distress even to mild metabolic imbalance.

In conclusion, a high concentration of sulfate in the drinking water may be an associative factor adversely affecting blood thiamine concentration in beef cattle. Apparently, a sub-population of beef cattle in Saskatchewan is predisposed to become marginally deficient or deficient in thiamine. Such a deficiency state is most likely to occur in areas where animals are exposed to high sulfate water. Considering the prevalence of the high sulfate water on the Canadian Prairies, these findings may be of economic importance. More research on thiamine metabolism in ruminant livestock exposed to high sulfate water is needed.

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**BIOCHEMICAL FEATURES IN A HERD OF CATTLE WITH INSTANCES  
OF POLIOENCEPHALOMALACIA ASSOCIATED WITH DRINKING  
HIGH SULFATE WATER: A FIELD STUDY**

**ABSTRACT**

A herd of 110 mature cattle and 100 calves at pasture were offered drinking water from a new well. The quality of the water was not checked. After consuming this water for approximately five weeks two cows died and nine developed neurological signs. Two of the affected cows were euthanised. Heads of three animals were submitted for pathological examination, all of which had brain lesions characteristic of polioencephalomalacia (PEM). Analysis of water revealed sulfate level of 7200 ppm. An investigation of blood thiamine indices was undertaken in the herd. Blood thiamine concentrations for all animals were slightly higher or within the range observed in normal cattle at pasture. However, low transketolase (TK) activity and high thiamine pyrophosphate (TPP) effect values indicated mild to moderate metabolic thiamine deficiency in some animals from both age groups. This was more prevalent in calves than in adults, although there were no incidences of PEM in calves. There were no differences in blood TK activity or TPP effects between affected and normal animals. Therefore, it appears that overt thiamine deficiency, based on currently used indices, is not a primary cause of sulfur induced PEM.

Approximately 27% of the cows and 11% of the calves had plasma copper (Cu) concentrations of  $<0.6$  mg/L. Serum sodium (Na) levels were slightly lower in two affected animals. However, potassium (K) concentrations were marginally high in clinically normal animals. Chloride (Cl) concentrations were marginally below reference levels in three affected animals. Serum magnesium (Mg) concentrations were either slightly lower or higher in two affected animals but normal in all others. Sodium, Cl and Mg levels were within reference values in clinically normal cattle. Serum calcium (Ca) concentrations were within normal limits in all animals of both groups. Serum phosphorus (P) concentrations were marginally higher in clinically normal animals. Serum creatinine and urea levels were within normal limits in clinically normal animals but four affected animals had markedly elevated urea levels and two had elevated creatinine levels. Aspartate aminotransferase (AST) activity was increased in two animals and creatinine phosphokinase (CK) activity was increased in five affected animals. Serum gamma-glutamyltransferase (GGT) activity was normal in all. No changes were noted in serum enzymes among clinically normal animals.

## INTRODUCTION

An increasing number of reports suggest that excess dietary sulfur<sup>a</sup> (S) is implicated in the pathogenesis of polioencephalomalacia (PEM). Polioencephalomalacia has been documented in cattle exposed to a high concentration of S in feed (Raisbeck 1982, Sadler et al. 1983, Hibbs and Thilsted 1983) and water (Harries 1987, Gooneratne et al. 1989a, Beke and Hironaka 1991). In addition, the disease has been experimentally induced by feeding excess dietary S to sheep (Gooneratne et al. 1989b) and cattle (Gould et al. 1991).

The recommended dietary S level (organic and inorganic forms) for beef cattle is 0.08 - 0.15% (National Research Council 1984), and dietary S above 0.3 - 0.4% may cause toxicity (Kandilis 1984). Dietary S at a level as low as 0.38% was reported to induce PEM in cattle (Gould et al. 1991).

Polioencephalomalacia most often affects younger stock (Sullivan 1985), being only rarely observed in mature animals. How the pathogenesis is related to age is not clear.

Retrospectively, PEM has been regarded as some form of thiamine deficiency syndrome (Loew 1975, Edwin and Jackman 1982, Blood and Radostits 1989). One of the strongest arguments for thiamine involvement was derived from the observation that in the majority of field cases of PEM (Pill 1967, Loew 1975, Dickie et al. 1979, Thomas 1986, McGuirk 1987) as well as in the cases likely associated with excessive intake of S (Harries 1987, Gooneratne et al 1989a, Khan

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<sup>a</sup>In ruminants, the term S may represent all forms of ingested sulfur since sulfur from dietary sources is reduced in the rumen to sulfide.

et al. 1987), thiamine appears to be an effective treatment. However, the role of thiamine in the pathogenesis of PEM remains obscure.

A high concentration of sulfate in the drinking water adversely affects blood thiamine status in beef cattle (Olkowski et al. 1991a) and low thiamine status may increase the risk of the disease in animals fed excess S (Olkowski et al. 1991b). However, it appears that overt thiamine deficiency is not the primary or sole etiological factor in S induced PEM (Gooneratne et al. 1989b, Gould et al. 1991, Olkowski et al. 1991b).

An increase in dietary S in ruminants limits the availability of Cu in the alimentary tract (Suttle 1974). Furthermore, sulfite ions, which are produced in the rumen by sulfate reducing bacteria (Lewis 1954), cause destruction of thiamine (Leichter and Joslyn 1969). Concurrent thiamine and Cu deficiencies can occur in feedlot cattle that consume water with a high sulfate content (Gooneratne et al. 1989a).

Little is known of the mechanisms or the biochemical changes associated with S-induced PEM in ruminants. It is not known whether or not there is any common etiology with other field cases of PEM or if the disease has any specific common diagnostic markers besides the similarity of lesions and clinical signs.

The objective of the present study was to examine blood thiamine and serum Cu status, serum electrolyte concentrations, and biochemical indices of liver, muscle and kidney damage during an outbreak of PEM in a herd of cattle, where the cause of the disease was associated with a high concentration of sulfate in the

drinking water. Of particular interest, was the study of trends in a large population of cattle exposed to high sulfate-water in terms of the interrelationships of commonly used indices of thiamine status and to compare the results between young and adult animals as well as between affected and clinically normal animals.

## **MATERIALS AND METHODS**

**Background.** The present study involved a herd of cattle (Angus/Hereford crossbred) comprised of 110 adult and 100 (3-4 months old) calves grazing summer pasture. The animals had free access to a mineral mix (Feed-Rite Limited, Winnipeg, Manitoba) containing required macro and micro elements. The investigation was undertaken following an outbreak of PEM in this herd. Only mature animals were affected. A total of 11 animals were diagnosed with PEM of which two died, two were euthanized and seven were routinely treated with thiamine and recovered. Detailed records of the doses and frequencies were not kept.<sup>b</sup>

A change in water source five weeks prior to the outbreak was the only identifiable factor that could be associated with the occurrence of the disease. Prior to its use, the quality of the water from the new well was not checked. The analysis of water performed during the investigation showed that the water from

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<sup>b</sup> The outbreak was attended by the Field Service, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada. The tentative diagnosis in vivo was confirmed by post mortem diagnosis made by Dr. E. Clark, Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.

new well contained 7200 ppm of sulfate.<sup>c</sup> This was in sharp contrast to the 750 ppm sulfate content of the water from the previous source. Since the intake of S in drinking water alone was considered to be at toxic level (NRC 1984), it has been inferred that the outbreak of PEM was associated with the exposure of the animals to high sulfate water. The use of high sulfate water was discontinued and no new instances of PEM were reported.

**Sampling and Measurements.** All animals were sampled on the third day of the outbreak. Serum electrolyte concentrations and biochemical indices of liver, muscle and kidney damage were measured in five clinically affected animals. The same measurements were also performed in five other clinically normal animals randomly selected from the herd. Plasma Cu concentration was measured in 85 adult cattle (including affected animals) and 94 calves. Blood samples were obtained from the jugular vein into glass vacutainers containing heparin (Becton Dickinson, Rutherford, NJ). Blood thiamine concentrations, TK activity and TPP effects were measured in 100 calves and 99 adults that were asymptomatic, and 6 adults that showed clinical signs characteristic of PEM but were not treated with thiamine prior to samples collection.

#### **Analytical procedures:**

**Blood Chemistry.** Measurement of serum concentrations of CK, AST, GGT, urea, Na, K, Cl, Ca, and Mg, were carried out using a Discrete Analyzer with

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<sup>c</sup> Water analysis was performed by the Saskatchewan Research Council, Saskatoon, SK, Canada, according to the method described in "Standard methods for the examination of water and waste water. 1985. American Public Health Association, 16th ed., Washington, DC."

Continuous Optical Scanning (DACOS, Coulter Electronics Inc., Hialeah, FL) in the Clinical Pathology Lab., WCVI.

**Copper analysis.** Plasma Cu concentrations were measured in the trichloroacetic acid (TCA) soluble fraction as outlined by Smith and Wright (1975). Copper was measured using an atomic absorption spectrophotometer (Model 5000; Perkin Elmer, Norwalk, CT).

**Blood Thiamine and TK analysis.** For measurement of TK activity and thiamine concentration, blood was hemolyzed by freezing at  $-20^{\circ}\text{C}$ . Blood hemolysates were centrifuged at 2000g for 15 min and the supernatants used for the assay. The TK assays were carried out as described by Massod et al (1971). The red blood cell (RBC) TK activities were expressed as  $\mu\text{mol sedoheptulose (SD) min}^{-1} \text{g}^{-1}$  hemoglobin (Hb). Hemoglobin concentration in blood hemolysates was determined using a kit method (Sigma diagnostic procedure No. 525, St. Louis, MO). The percentage increase in TK activity after addition of thiamine pyrophosphate (TPP) to the assay was expressed as the TPP effect. Thiamine was measured as described by Olkowski and Gooneratne (1991, see appendix).

#### **Statistics:**

Statistical analysis was carried out using the Student t test (Snedecor and Cochran 1989). The interrelationships between thiamine and TK, thiamine and TPP effects, and TK and TPP effects were established using regression analysis. Statistical significance was assumed when the probability of making a type I error was less than 0.05.

## RESULTS

**Gross pathology and histopathology.**<sup>d</sup> Briefly: The brains in the three necropsied animals showed the dorsal surfaces of the gyri of the occipital, parietal, and to a lesser extent the frontal lobes to be bilaterally and multifocally flattened and yellowish. The brains were soft but did not show tentorial herniation or cerebellar coning typical of severe brain swelling.

On the cut surfaces, the sides and depths of the cerebral cortical sulci were multifocally yellowish and occasionally fluoresced under the ultraviolet light of a Wood's lamp (Fig.1). Bilaterally, the central thalamic and mesencephalic (midbrain) regions contained large areas of hemorrhage, in two cases being up to two cm diameter. These hemorrhagic foci were also consistently present in the colliculi of the midbrain and in one case extended into the medulla oblongata. In two cases, petechial hemorrhagic foci were also scattered through the involved cortical areas. Small hemorrhagic foci also extended cranially from the thalamus into the basal ganglia and internal capsules. Multiple routine sections of all brains showed bilateral and multifocal, acute cortical grey matter necrosis, most consistently involving outer cortical laminae. These foci were most abundant in the parietal and occipital regions. The necrotic lesions were well delineated from the surrounding, most consistently involving the bottoms and sides of the sulci. However, in some areas the full thickness of the cortex was involved, including the tops of the gyri.

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<sup>d</sup> The description of pathological findings was graciously provided by Dr. E. Clark, Department of Veterinary Pathology, WCVN.



Fig. 1. Sections of brain from a cow exposed to drinking water containing 7200 ppm of sulfate. The cow developed clinical signs of PEM during the fifth week of exposure. Note multiple malacic areas and hemorrhage in various parts of the brain. (Courtesy of Dr. E. Clark)

**Serum electrolytes.** Serum Na levels were slightly lower in two affected animals (Table 1). On the other hand, K concentrations were marginally higher ( $6.1 \pm 0.2$  mmol/L) than the reference limits (3.9 - 5.9 mmol/L) in clinically normal animals. Chloride concentrations were marginally below reference levels (96 - 110 mmol/L) in three affected animals. Serum Mg concentrations were either slightly lower or higher in two affected animals but normal in all others. Sodium, Cl and Mg levels were within the reference range in clinically normal animals. Serum Ca concentrations were within normal limits in all animals of both groups. Serum P concentrations were marginally higher ( $2.86 \pm 0.35$  mmol/L) in clinically normal animals. Among affected animals, only one cow had a serum P (0.52 mmol/L) below reference values (1.08 - 2.76 mmol/L).

**Serum creatinine and urea.** Serum creatinine and urea levels were within normal limits in clinically normal animals but four affected animals had mildly elevated urea levels and two had elevated creatinine levels.

**Serum enzymes.** Aspartate aminotransferase activity was increased in two affected animals and CK activity was increased in four affected animals (Table 1). The changes of the latter enzyme were most marked in terminally ill cases. No changes were noted in serum enzymes among clinically normal animals.

**Plasma Cu Status.** Plasma Cu status differed ( $p < 0.003$ ) between young and mature animals with respective means ( $\pm$  SD) being  $0.71 \pm 0.13$  and  $0.66 \pm 0.12$  mg/L. The distribution of plasma Cu concentrations in young and mature animals is shown in (Fig.2).

Table 1. Serum electrolyte and urea concentrations and serum activities of creatine kinase (CK), aspartate amino transferase (AST) and gamma-glutamyl transferase (GGT) in cattle drinking water containing 7200 ppm sulfate diagnosed with polioencephalomalacia.

	Case				
	1 <sup>e</sup>	2 <sup>e</sup>	3 <sup>t</sup>	4 <sup>t</sup>	5 <sup>t</sup>
Sodium	135	134 <sup>l</sup>	135	130 <sup>l</sup>	141
Potassium	3.9	4.8	4.6	4.5	4.8
Chloride	93 <sup>l</sup>	100	93 <sup>l</sup>	93 <sup>l</sup>	96
Magnesium	1.48 <sup>h</sup>	1.32	1.04	0.99	0.73 <sup>l</sup>
Calcium	2.19	2.18	2.56	2.23	2.38
Phosphorus	1.54	0.52	2.61	1.83	2.43
Urea	12.0 <sup>h</sup>	5.9	8.3 <sup>h</sup>	9.9 <sup>h</sup>	19.0 <sup>h</sup>
Creatinine	288 <sup>h</sup>	139	169	162	230 <sup>h</sup>
CK	15696 <sup>h</sup>	1185 <sup>h</sup>	217	681 <sup>h</sup>	391 <sup>h</sup>
AST	420 <sup>h</sup>	125 <sup>h</sup>	98	104	71
GGT	25	25	18	21	17

e euthanized, t treated/recovered

h values exceeding upper reference, l values below lower reference

Reference values as determined by the Clinical Pathology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan are: for electrolytes sodium, potassium, chloride, magnesium, calcium and phosphorus being 135 to 151, 3.9 to 5.9, 96 to 110, 0.8 to 1.32, 2.11 to 2.75 and 1.08 to 2.76 mmol/L respectively, for urea <7.5 mmol/L, for creatinine 67 to 175 µmol/L and for enzymes CK, AST and GGT <350, 46 to 118 and <31 U/L respectively.

It is important to note that the reference range is a mean  $\pm$  2 SD, thus 5% of normal animals will be outside lower and upper limits.

The data (cases 1 through 4) compiled in this table was obtained from the clinical records W.C.V.M. graciously provided by Dr. E. Janzen.

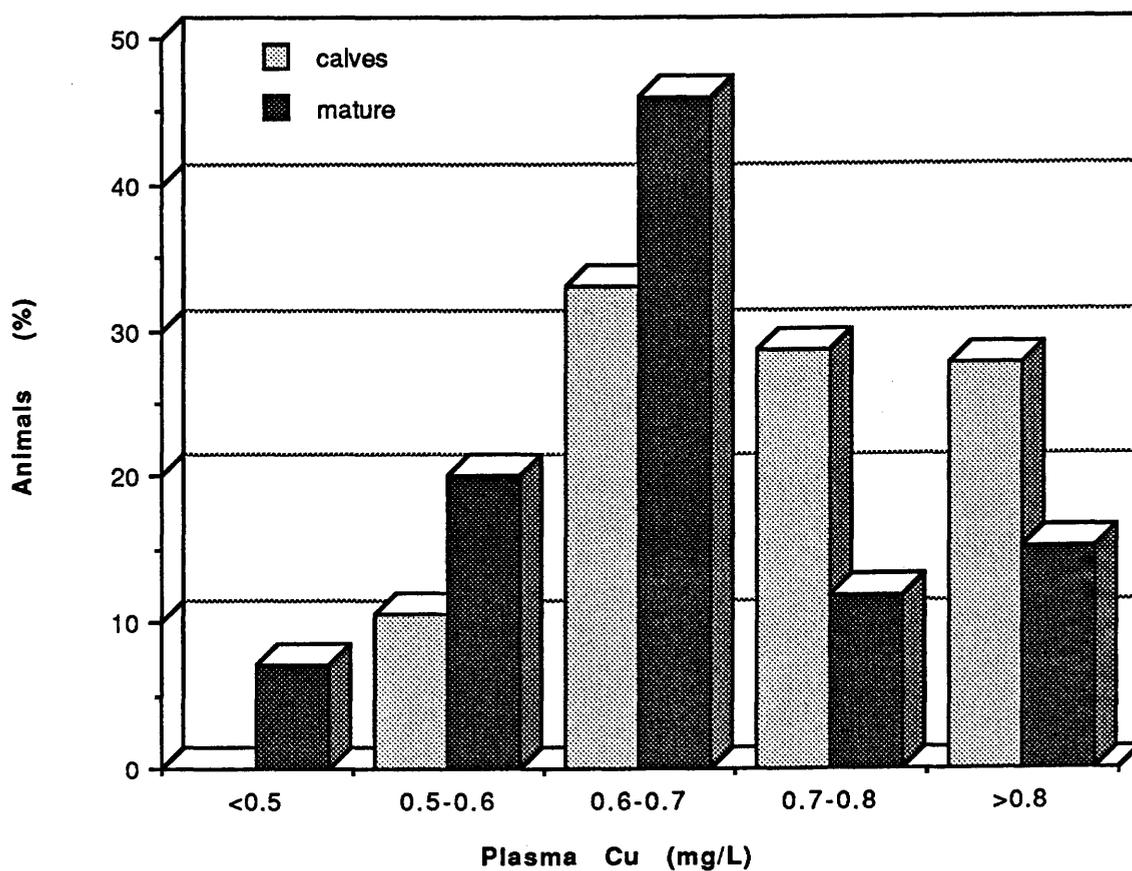


Fig. 2. Plasma copper concentrations in calves and cows exposed to drinking water containing 7200 ppm of sulfate. Approximately 27% of cows and 11% of calves had plasma Cu concentrations of <0.6 mg/L. Overall, calves had higher ( $p<0.003$ ) plasma Cu concentrations than mature animals.

Among adults, 7.1% had plasma Cu concentrations of 0.4 to 0.5 mg/L. Further, 20% of mature animals and 11% of calves had plasma Cu concentrations of 0.5 to 0.6 mg/L. Plasma Cu concentrations in clinically affected animals were higher ( $p < 0.017$ ) than in clinically normal animals with respective means ( $\pm$ SD) being;  $0.79 \pm 0.11$  and  $0.64 \pm 0.11$ .

**Thiamine Status, TK Activity and TPP Effect.** The distribution of blood thiamine concentrations varied substantially between calves and mature cattle (Fig. 3). Blood thiamine levels of calves were higher ( $p < 0.001$ ) than those of mature animals, with means ( $\pm$  SD) being  $60.4 \pm 12.2$  and  $45.8 \pm 12.1$   $\mu\text{g/L}$ , respectively. Blood thiamine concentrations in animals diagnosed with PEM were higher ( $p < 0.01$ ) in comparison to clinically normal animals (Table 2).

There were no differences in TK activity or TPP effects between affected and normal cows (Table 2). Also, calves and adults had similar TK activities, but TPP effects were higher ( $p < 0.001$ ) in calves. The TK activities and TPP effects (mean  $\pm$  SD) were: (TK)  $0.44 \pm 0.15$  and  $0.41 \pm 0.11$   $\mu\text{mol sedoheptulose min}^{-1} \text{g}^{-1} \text{Hb}$ ; (TPP effect)  $15.3 \pm 15.7$  and  $38.7 \pm 23.2\%$ , in adults and calves respectively. The distribution of TK values was similar in calves and mature animals (Fig.4). On the other hand, the distribution of the TPP effects varied considerably between calves and mature animals (Fig.5).

The interrelationships among thiamine, TK and TPP effects for adults and calves are shown in Figs. 6 and 7 respectively. The regression lines demonstrated some trends indicating interdependence of these parameters for both groups.

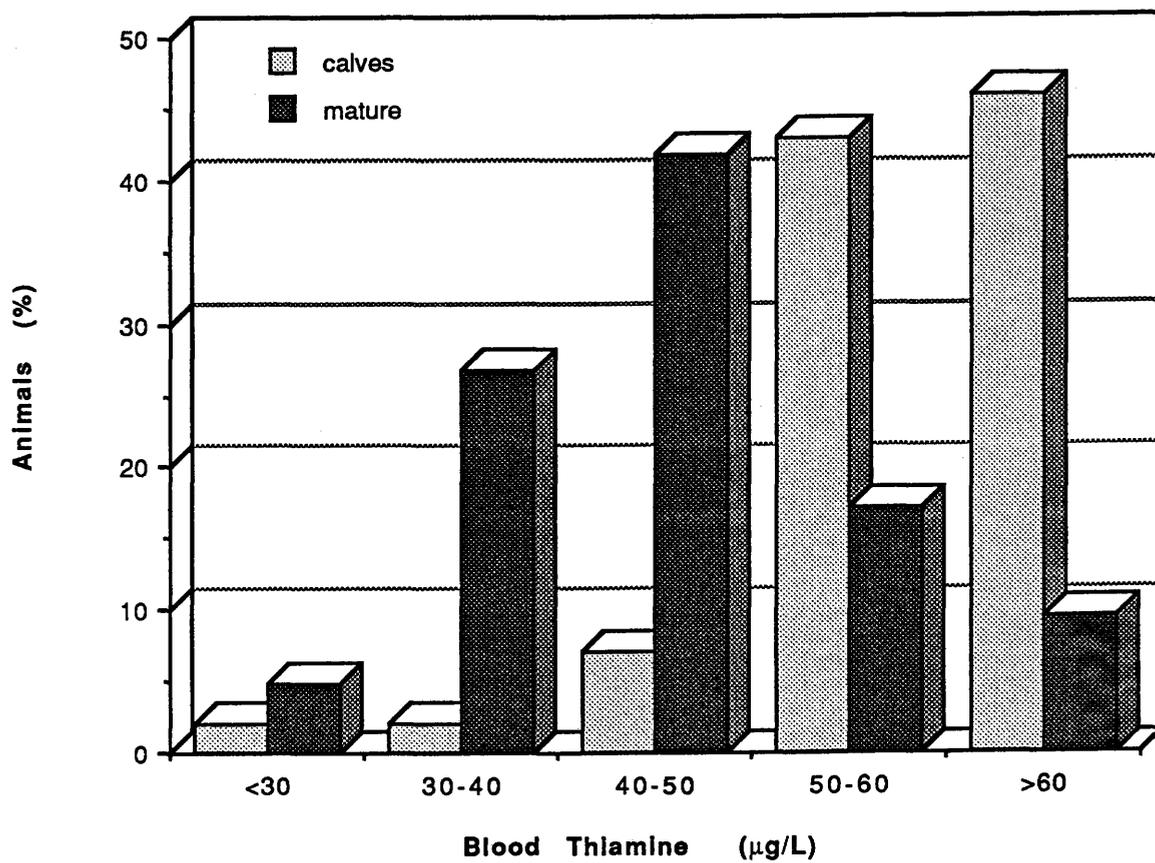


Fig. 3. Blood thiamine concentrations in calves and mature animals exposed to drinking water containing 7200 ppm of sulfate. Blood thiamine levels of calves were higher ( $p < 0.001$ ) than those of mature animals.

Table 2. Comparison of blood thiamine concentrations, erythrocytes transketolase (RBC TK) activities, and TPP effects in cattle diagnosed with polioencephalomalacia and clinically normal. All animals were exposed to drinking water containing 7200 ppm of sulfate.

	Thiamine ( $\mu\text{g/L}$ )	TK ( $\mu\text{mol SD min}^{-1} \text{g}^{-1} \text{Hb}$ )	TPP Effect (%)
Calves n=100 (Asymptomatic)	60.4 $\pm$ 12.2 *	0.41 $\pm$ 0.1	38.2 $\pm$ 23.2 *
Adults n=99 (Asymptomatic)	44.8 $\pm$ 11.4	0.44 $\pm$ 0.2	15.1 $\pm$ 15.9
Adults n=6 (PEM)	62.7 $\pm$ 11.1 *	0.54 $\pm$ 0.1	21.4 $\pm$ 12.2

Values expressed as means  $\pm$  SD.

\*  $P < 0.01$  from asymptomatic adults

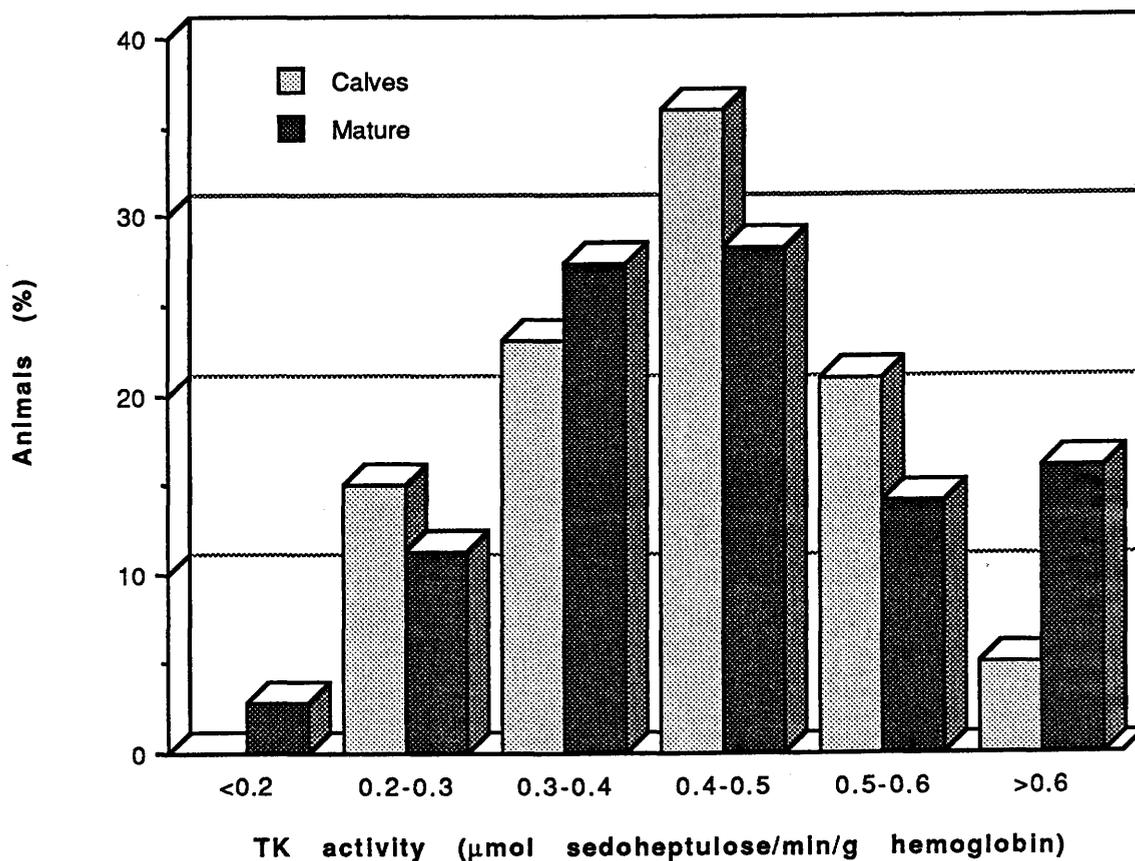


Fig.4 Blood transketolase (TK) activities in calves and adults exposed to drinking water containing 7200 ppm of sulfate. The distribution of TK values was similar in calves and mature animals. Approximately 14% of adults and 15% of calves had TK activities less than 0.30  $\mu\text{mol sedoheptulose min}^{-1} \text{g}^{-1} \text{Hb}$ .

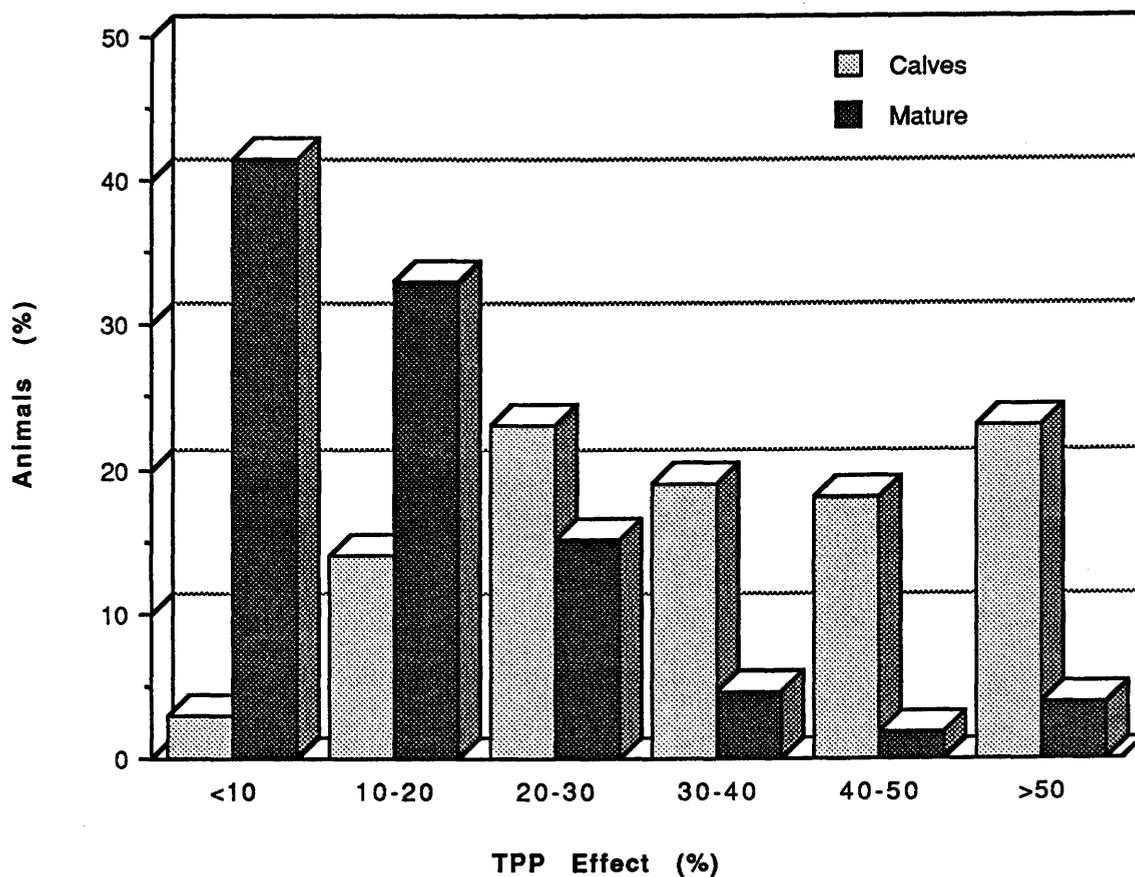


Fig. 5. The distribution of TPP effects in calves and adults exposed to drinking water containing 7200 ppm of sulfate. Approximately 10% of adults and 60% of calves had TPP values exceeding 30%.

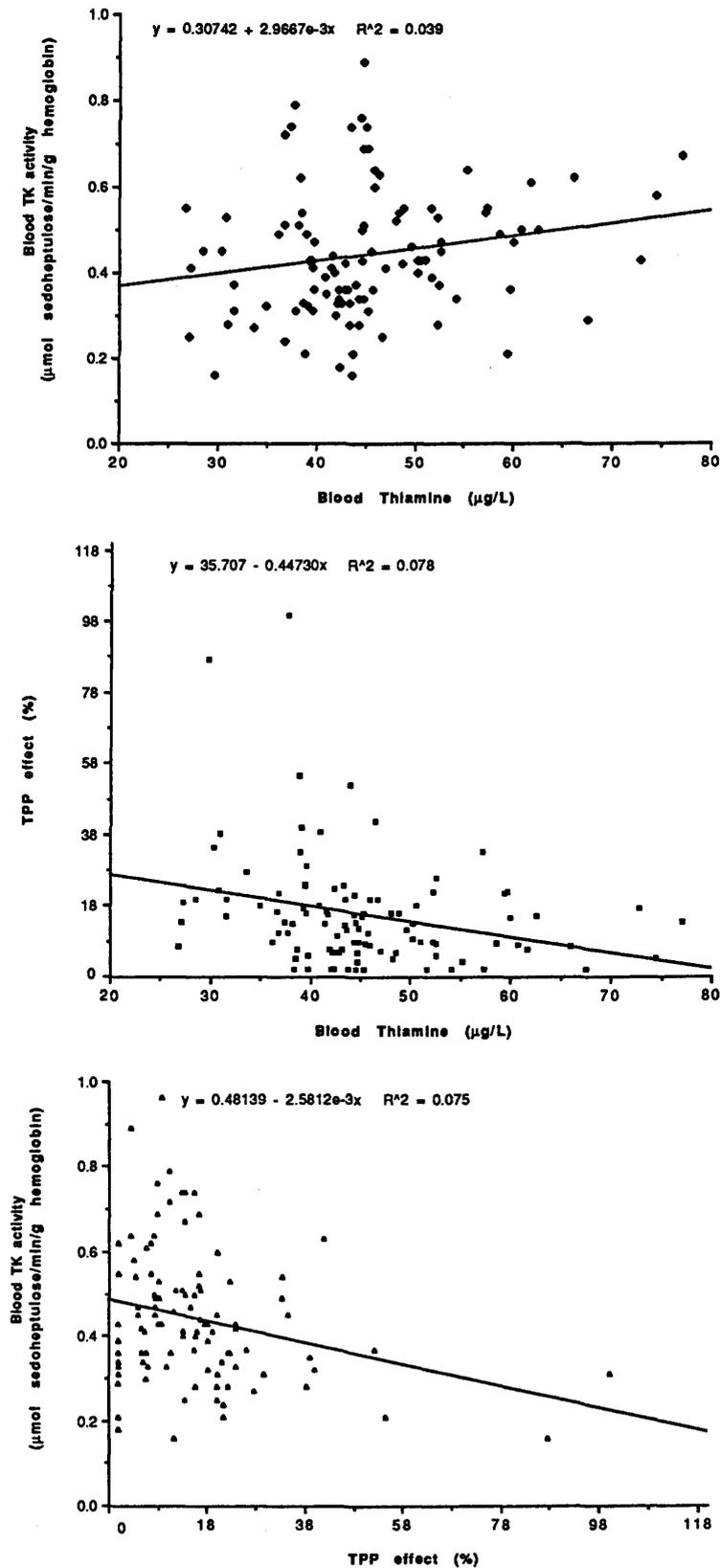


Fig. 6. Regression lines depicting the relationships among blood thiamine concentration, transketolase (TK) activity, and TPP effects in mature cattle exposed to drinking water containing 7200 ppm of sulfate.

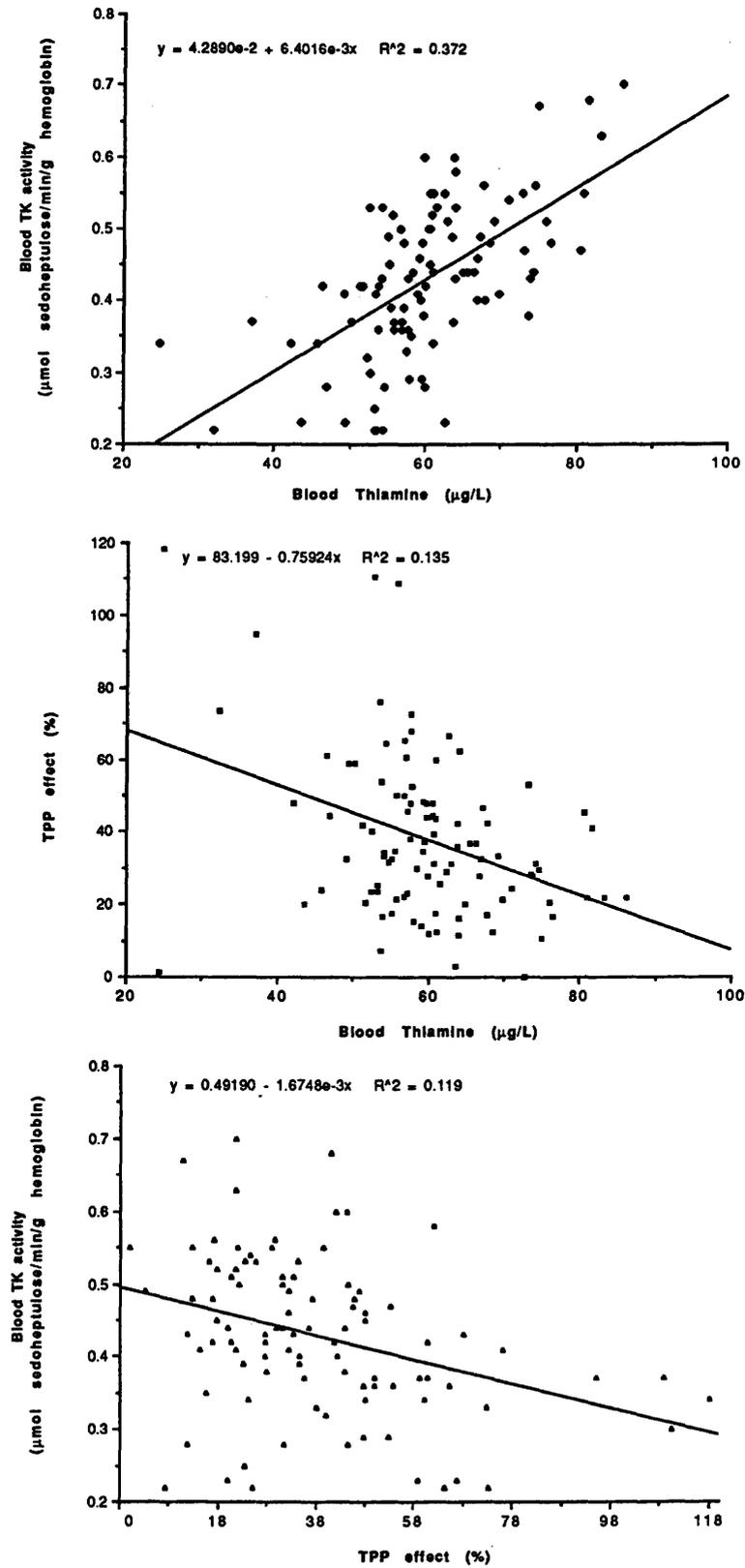


Fig. 7. Regression lines depicting the relationships among blood thiamine concentration, transketolase (TK) activity, and TPP effects in calves exposed to drinking water containing 7200 ppm of sulfate.

## DISCUSSION

The gross and histopathological lesions seen in the present study differ in some ways from other PEM cases seen in Western Canada. The severe vascular lesions seen in the midbrain and thalamus, and less prominently lesions in other brainstem and cortical regions of these cases, are not typical of the bovine PEM, although the acute lesions as in the cortices have been seen in similar subcortical locations in other PEM cases (Clark, personal comm.). It is not known if there is any cause-effect relationship or metabolic link between the vascular and malacic lesions.

Normally, the incidence of PEM is highest in younger stock (6-8 months of age) (Sullivan 1985). Notably, in the present study the disease occurred only in mature animals.

High serum CK values are usually indicative of either an increase in muscle cell permeability or acute damage to the muscle. However, CK levels may also be of diagnostic value in CNS disorders (Kaneko 1980). Increases in the serum CK activity have been reported in PEM of sheep (Smith and Healy 1968) and in amprolium-induced PEM in calves (Lilja 1973). Increases in serum CK have been reported in a variety of muscular disorders including Se deficiency-associated nutritional muscular dystrophy in lambs (Boyd 1976). Muscular dystrophy has been reported in sheep fed high S diets (Ganther and Bauman 1962). However, Khan et al. (1987) did not find any alterations of Se indices in cattle fed high sulfate diets. The method used in the present study for CK analysis does

not distinguish organ-specific CK isoenzymes, but the increased serum CK as observed in the present study may be indicative of CNS damage being the cause of CK release.

In the present study the increase in serum AST and Mg, but not in CK was observed only in one animal. Serum AST was markedly elevated in two recumbent animals (cases 1 and 2) indicating damage to muscle. Lilja (1973) reported only a slight increase in AST and a marked increase in CK, but no changes in serum Mg in amprolium-induced PEM in calves. However, hypermagnesemia was observed in sheep treated with amprolium (Loew and Dunlop 1972a).

Lowered serum K has been reported in calves with PEM (Clegg 1966). However, hypokalemia did not occur with amprolium induced PEM in ewes (Loew and Dunlop 1972a) or calves (Lilja 1973). There were no changes in serum K concentration in affected animals in the present study, but it is notable that the K level in the clinically normal group was marginally higher. Increased serum K concentrations have been reported in amprolium-treated geldings during the onset of clinical signs (Cymbaluk et al. 1978). Serum Na and Cl were only marginally lower in some affected animals. Decreased serum Na and Cl were reported in heifers exposed to elemental S (Gunn et al. 1987). The clinical significance of this is uncertain.

Some of the affected animals had increased serum creatinine and urea levels. Elevated serum urea levels were reported in acute S toxicity in heifers (Gunn et

al. 1987). However, it is unlikely that the alterations seen in the present study are attributed to the effects of S seen in acute S toxicity.

The detrimental effect of S on Cu metabolism is well documented from both experimental (Suttle 1974, Allen and Gawthorne 1987) and field studies (Smart et al. 1986, Gooneratne et al. 1989a). In the present study a mineral mix containing 2600 ppm Cu was offered to the animals on a free choice basis. However, approximately 27% of cows and 11% of calves had plasma Cu concentrations of  $\leq 0.6$  mg/L. A level below 0.6 mg/L is considered to be marginally inadequate by some workers (Puls 1988). In the present study, depigmented hair noted in several animals would be consistent with mild hypocupremia.

The Cu status of an animal may be of importance for thiamine metabolism in animals exposed to excess dietary S (Olkowski et al. 1991c). In ruminants, the antagonism between Cu and S appears to be due to the formation of cupric sulfide (CuS) in the GI (Suttle 1974). Perhaps, this complexing of S into CuS could be considered as a possible mechanism protecting thiamine against S. This is consistent with the findings of Gooneratne et al. (1989a) where lowered status of both Cu and thiamine observed in beef cattle drinking water with excess S was corrected by supplementation of Cu alone.

In the present study, all animals had blood thiamine concentrations moderately higher or within the range observed in normal cattle at pasture (Hill et al. 1991). This is somewhat different from our previous observation (Olkowski et al. 1991a) where a part of a cattle population drinking high sulfate-water had

blood thiamine concentrations indicative of deficiency. It has to be stressed, however, that the water sulfate level in the present study was 7200 ppm, i.e. approximately double the highest level observed in the previous survey. This apparent discrepancy in blood thiamine concentrations may be partially attributed to differences in the feeding regimes (pasture vs feedlot). However, the results of the present study indicate that the apparently normal blood thiamine levels may not adequately reflect the metabolic requirements for thiamine in young animals or in animals exposed to excess dietary S.

Measurements of blood TK activities and TPP effects are commonly accepted as indices of thiamine status of animals (Bogin et al. 1985, Ali et al. 1987). However, there is a lack of a standardized reference TK activity or TPP effect values for cattle. The classification for degrees of thiamine deficiency developed for humans does not seem to be applicable for ruminants (Edwin et al. 1979, Bogin et al. 1985). Further, it appears that the TPP effect values may vary substantially depending on breed and/or type of production (Bogin et al. 1985). Based on several citations (Quaghebeur et al. 1974, Thornber et al. 1981, Mueller and Asplund 1981, Bogin et al. 1985) and our own observations, it appears that in general terms, TK activities below  $0.30 \mu\text{mol sedoheptulose min}^{-1} \text{g}^{-1} \text{Hb}$  and/or TPP effect above 30% may be indicative of metabolic thiamine inadequacy. Using the above standards, some 10% to 15% of adults and up to 60% of calves in the present study should be considered deficient.

It must be stressed that the concept of metabolic deficiency was derived from observation of metabolic changes which occur during thiamine deprivation. However, in thiamine deficiency there is no clear association between metabolic changes and CNS disorder (Brin 1962, Mueller and Asplund 1981). In addition, a wide range of TK and TPP effect values are observed in animals affected with PEM as well as in animals showing no nervous signs (Roberts and Boyd 1974, Quaghebeur et al. 1974, Bogin et al. 1985).

It is noteworthy that calves and adults had similar TK activities, but blood thiamine levels and TPP effects were higher ( $p < 0.001$ ) in calves. Hence, somewhat paradoxically, despite higher blood thiamine levels, more calves than adult animals had TPP effect values indicative of metabolic thiamine deficiency. Interestingly, despite an apparent metabolic thiamine deficiency not one calf succumbed to the disease, although PEM is usually seen in younger stock (Sullivan 1985). Likely, the higher blood thiamine concentration and the absence of morbidity in calves can be attributed to the fact that the calves, benefiting from the milk of their dams, were less dependent on the contaminated water. However, a higher incidence of elevated TPP effect values in calves may indicate that the requirement for thiamine was considerably higher in calves than in mature cattle.

Low TK activities and/or elevated TPP effect values seen in several mature animals from this herd could be indicative of mild to moderate metabolic thiamine deficiency. However, the changes in these indices, indicating the state

of thiamine deficiency, were not manifested in clinically affected animals. There were no significant differences in TK activities or TPP effects between affected and normal cows. Thus, the present data indicates that changes in the blood thiamine status indices cannot be correlated with the incidence of PEM. This is in accord with findings of Sager et al. (1990) and Gould et al. (1991).

The reports on thiamine deficiency in animals affected with PEM are conflicting and thus far numerous studies on the pathogenesis of PEM have not produced conclusive evidence that overt thiamine deficiency is indeed involved. It is not known whether S induced disease and other cases of PEM have a common etiology. However, the existing studies on S induced PEM (Gooneratne et al. 1989b, Gould et al. 1991, Olkowski et al. 1991b) indicate that overt thiamine deficiency (as it is currently interpreted) is not the primary cause of the disease. Also, the present data demonstrates that the etiology of S induced PEM cannot be explained by metabolic changes originating from depletion of thiamine. In true cases of thiamine deficiency, blood thiamine indices are the first to be affected (Brin 1962).

Notably, there were poor associations among blood thiamine indices. The regression lines depicting the interrelationships among blood thiamine, TK activities, and TPP effects for adults (Fig.6) and calves (Fig.7) demonstrated some trends indicating interdependence of these parameters for both groups, but the overall correlations were rather poor. Analysis of individual data points from Figs. 6 and 7 shows that a number of animals had "normal" blood thiamine

concentrations coinciding with elevated TPP effect values. In addition, in several instances, low TK activity occurred concurrently with "normal" blood thiamine concentrations, and TPP effects were not apparent in these assays. The above situation fits the scenario of increased metabolic demand resulting possibly from increased requirement and/or hindered bioavailability of the vitamin (Loew 1975).

Poor correlation among thiamine status indices and/or absence of lowered blood thiamine in animals with PEM are noticeable in the older literature, but this was often attributed to "lack of specificity of methods for thiamine analysis" (Loew and Dunlop 1972, Quaghebeur et al. 1974, Rammell and Hill 1986). However, Sager et al. (1990) and Gould et al. (1991) who used the HPLC method did not observe thiamine depletion and/or changes in TK or TPP effect values in affected animals. Further, the thiamine measurements in the present study were performed using a highly specific and sensitive biological method assaying the biologically available thiamine (Baker and Frank 1968).

The increased metabolic demand for thiamine may be independent from the requirement of TPP dependent enzymatic activities. Sager et al. (1990) did not observe changes in TK and TPP effect values in PEM affected calves, yet the animals responded to parenteral treatment with thiamine.

Thus, it appears that the therapeutic effect of thiamine may be other than supplying the TPP co-factor for enzymatic activity, i.e. thiamine may have some unknown "protective/sparing" effects, possibly of pharmacological nature. This is consistent with our previous observation (Olkowski et al. 1991b).

Gould et al. (1991) postulated that the lesions of PEM may be related to a profuse synthesis of sulfides in the rumen of animals exposed to excess dietary S. The possible involvement of S metabolites in the pathogenesis of S induced PEM was also raised by our group (Gooneratne et al. 1989, Olkowski et al. 1991b). It is noteworthy that the excess dietary S in ruminants produces lesions in the brain, a feature unseen in other animals, but also the metabolism of S is unique in ruminants. Hence, it is conceivable that S metabolites produced in the rumen, absorbed and modified systemically and/or locally in the brain, when in excess, may have great injurious potential. Brain tissue, because of its high lipid content, would be extremely susceptible to oxidative damage inflicted by S metabolites such as sulfides, sulfites and/or their more reactive (free radicals) derivatives. The possibility of synthesis of S derived free radicals (in the rumen and/or systemically) and their injurious potential is supported by the work of Mottley and Mason (1988) and Ozawa and Hanaki (1990). Thus, the involvement of some form of S metabolites, likely derived from sulfides and/or sulfites produced in the rumen, in the brain injury seems a plausible hypothesis.

The role of thiamine in the pathogenesis of S induced PEM is not completely understood. At present, there is no alternative hypothesis explaining thiamine involvement in the PEM, and it appears that overt thiamine deficiency is not an etiological factor (Sager et al. 1990, Gould et al. 1991, Olkowski et al. 1991b). However, the condition appears to be responsive to thiamine therapy (Harries 1987, Gooneratne et al 1989a, Khan et al. 1987, Beke and Hironaka 1991, Olkowski

et al. 1991b). The data from the present study lends a support to these findings.

Interestingly, cattle (Goetsch and Owens 1987) and sheep (Olkowski et al. 1991b) fed excess dietary S tend to have higher blood thiamine concentrations than control animals. A similar tendency is also observed in animals showing clinical signs of PEM (Gooneratne et al. 1989b, Gould et al. 1991). In the present study, the blood thiamine concentrations were higher than those observed under normal conditions (Hill et al. 1988). Thus, the ruminant host exposed to excess dietary S appears to maintain increased level of circulating thiamine. This may be a feature of an increased requirement for thiamine in animals exposed to excess dietary S.

The increased demand for thiamine in S toxicity, apart from the TPP requirement, may be considered in the context of detoxifying processes. It is noteworthy that the thiamine molecule may have natural features enabling a protective function against S metabolites. Free thiamine may be protective by scavenging free radicals (Theron et al. 1981). Also, thiamine in its thiazole open form with the exposed -SH group could serve as a conjugate to detoxify sulfide derivatives. Further, the readiness of thiamine to react with sulfite ions (Leichter and Joslyn 1969) may indeed be considered as a detoxifying process. These processes would be at the expense of destroying the thiamine molecule, with possible consequences of secondary metabolic deficit. Hence, the hypothesis of increased requirement for free thiamine (independent from the requirement of TPP dependent enzymes) in S toxicity seems plausible.

In summary, the lesions in S-induced PEM appear to be qualitatively similar to other cases, but there are distinctive features not observed in other cases. The comparison between S induced PEM in the present study and PEM cases reported in the literature was somewhat difficult since there is no comprehensive study compiling all biochemical parameters discussed here. Furthermore, the fragmented information collected from a variety of different studies is not entirely consistent. Some biochemical parameters observed in high S-related PEM in cattle appear to be similar to those observed in naturally occurring and amprolium-induced PEM in ruminants. No pathognomonic diagnostic markers enabling early recognition of the S induced PEM were found among the most commonly used clinical chemistry parameters. The alterations of the parameters in some animals can be attributed to the clinical status rather than to the etiological factors. Changes in serum electrolytes were only marginal and did not occur consistently.

Sulfur-related PEM in cattle appears to be thiamine responsive, but there was no evidence of overt thiamine deficiency, based on commonly used indices of thiamine status, in affected animals. However, the signs of metabolic thiamine deficiency were quite obvious in some unaffected animals. Undoubtedly, the pathogenesis is complex, and the lesions caused by toxic effects of S are likely due to a combination of several factors. Involvement of S metabolites (as a primary cause of the lesions), rather than thiamine deficiency per se, seems a plausible hypothesis. Probable factors involved in the pathogenesis of S related PEM may

include the deficit of thiamine due to an increased demand for thiamine in response to toxic effects of S, which can be further hampered by interference of some unknown factor with thiamine bioavailability. Since the metabolic thiamine deficiency was evident in animals with "normal" blood thiamine concentrations, a revision of thiamine adequacy standards in young animals and in animals exposed to high dietary S is in order.

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## CHAPTER II. IN VIVO STUDIES

1. Effects of high dietary sulfur on brain functions using evoked potentials technique. A.A. Olkowski, S.R. Gooneratne, E.C. Crichlow, C.G. Rousseaux and D.A. Christensen. *Can. J. Vet. Res.* 1990, Vol. 54:113-118.
2. Sulfur-induced polioencephalomalacia in sheep: Some biochemical changes. S.R. Gooneratne, A.A. Olkowski and D.A. Christensen. *Can. J. Vet. Res.* 1989, Vol. 53:462-467.
3. The role of thiamine status in sulphur induced polioencephalomalacia in sheep. A.A. Olkowski, S.R. Gooneratne, C.G. Rousseaux and D.A. Christensen. *Res. Vet. Sci.* 1992. 52:78-85.
4. The effects of copper status on thiamine metabolism in sheep fed a high sulfur diet. A.A. Olkowski, S. R. Gooneratne, and D.A. Christensen. *Can. J. Anim. Sci.* 1991, Vol. 71:813-824.

## EFFECTS OF HIGH DIETARY SULFUR ON BRAIN FUNCTIONS USING EVOKED POTENTIALS TECHNIQUE

A.A. Olkowski, S.R. Gooneratne, E.C. Crichlow, C.G. Rousseaux and D.A. Christensen. *Can. J. Vet. Res.* 1990, Vol. 54:113-118.

### ABSTRACT

Brain stem auditory-evoked response (BAER) is a noninvasive technique used for detecting neurophysiologic abnormalities of the brain stem along the auditory pathway. BAER recordings were obtained from subcutaneous skin electrodes from two control sheep and 22 other sheep fed high sulfur (S) diets with low or high concentration of thiamine, copper (Cu), and molybdenum (Mo). At least four peaks (I,II,III,IV) of varied amplitude were observed in all animals. Neurophysiologic abnormalities due to decreased conductivity and/or excitability of nerve fibers along the auditory pathway were found on the BAER recordings of sheep fed high S diet. Abnormalities of peaks and interpeak latencies within BAER recordings were related to histopathological observations of brain stem lesions. Lesions in the areas of the cochlear nuclei and lateral lemniscus were seen in conjunction with altered BAER components. However, abnormalities in BAER recordings and lesions in the brain stem also occurred in the absence of overt clinical signs. Analysis of interpeak latencies between peaks I and IV revealed significant differences among dietary groups. Sheep given diets low in Cu, Mo, and thiamine were affected most. Factorial analysis indicated thiamine and interactions among Cu, Mo, and thiamine as significant factors influencing interpeak latencies.

## INTRODUCTION

Copper (Cu), sulfur (S), molybdenum (Mo), and thiamine are considered essential nutrients for animals. However, when these nutrients are imbalanced in the diet, a complex array of interactions often leads to metabolic disturbances. Excess Mo and S in the diet decrease the availability of dietary Cu in ruminants (1). Recent reports have suggested a possible causal relationship between high S intakes, thiamine deficiency, and polioencephalomalacia (PEM) in cattle (2 - 5). Both thiamine (6) and Cu (7) are indispensable for brain metabolism. Hence a deficiency of either may lead to serious alterations of the normal function. Studies on early subclinical effects of essential nutrient deficiency on brain in vivo are few and the metabolic mechanisms of these deficiencies remain unclear.

Measurement of evoked potentials (EP) is a noninvasive, in vivo technique widely used in human medicine (8,12). One of the commonly used EP techniques is brain stem auditory evoked potential (BAER). It provides a noninvasive, reproducible and objective measure of brain stem function with a record of activity of the auditory pathway, reflecting electrical events ascending within the brain stem. BAER has been found to be extremely useful in the study of clinical neuropathology (8), particularly in the evaluation of neurologic disorders caused by demyelination, anoxic-ischemic encephalopathy and degeneration of neural tissue (8 - 12).

In recent years the EP technique has also interested veterinary investigators (13 - 15). Since it is a noninvasive technique it can be used to monitor the

progress of brain lesions and/or response to treatment. In this study the BAER was used to evaluate the effects of high dietary S on brain physiology. Morphological abnormalities of the regions examined electrophysiologically were examined using histopathological techniques.

## **MATERIALS AND METHODS**

**Animals and diets.** Twenty-four, 2 month-old cross-bred female sheep weighing 12-15 kg were housed in individual metabolism units. The basal diet was a pelleted barley (59%) - soybean meal (5%) - alfalfa (32%) mixture prepared to meet the minimum nutrient requirements recommended by National Research Council (NRC) (16). The diet contained 0.63% of S with two levels of each of Cu, Mo, and thiamine (see Table I). This diet was fed to 22 sheep. The remaining two sheep received normal S (0.19%) and 7.9, 4 and 234.0 mg kg<sup>-1</sup> DM of Cu, Mo and thiamine respectively. All animals had free access to water throughout the experiment and were offered the diets at rate of 1 kg per animal per day for 14 weeks.

Care of animals was performed in accordance with the guidelines presented in the Guide to the Care and Use of Experimental Animals (17,18).

**Baer recordings.** The BAER recordings were carried out on all sheep during the 14th week of experiment. Recordings from four (two from diet 1, one from diet 2, one from diet 4) of the 22 high S fed animals were also obtained on the 8th week of the experiment. Prior to BAER recording, animals were injected with sodium pentobarbital intravenously (iv) (20 mg/kg body weight) to maintain

them in a state of light anesthesia throughout the recording period. Sheep were positioned in sternal recumbency and testing was performed in a quiet room. The BAER recordings were obtained from three platinum Grass E2B subdermal needle electrodes (Grass instrument Co., Quincy, Mass). The active recording electrode was inserted subcutaneously just below the most ventral and caudal aspect of the pinna of the ear being tested and the reference electrode was likewise inserted at the vertex. The ground electrode was inserted subcutaneously in the untested ear, in a location similar to that of the active recording electrode. These recording electrodes were plugged into a SW-100A Electrode Selector (Nicolet Biomedical Instruments, Madison, WI) which was connected to a HGA-200A Physiological Amplifier (Nicolet Biomedical Instruments, Madison, WI) with the gain set at  $10^4$ . The output of the HGA-200A amplifier was then fed into a Clinical Averager (CA-1000). In recording the BAERs, the CA-1000 sensitivity was set at  $10 \pm 0.1 \mu\text{V}$ , the low end of the input bandpass filter was set at 30 Hz and the high end at 3000 Hz. The analysis time was 10 msec and the number of responses averaged was 2000.

**Auditory stimulation.** Auditory stimulation was obtained from a Nic-10007A Click Stimulator/Noise Masking Module (Nicolet Biomedical Instruments, Madison, WI). The test stimulus consisted of a 100  $\mu\text{m}$  condensation click of 90 dB intensity. This auditory stimulus was given at a rate of 11.1/sec into the ear under test whereas the untested ear received masking white noise at an intensity

of 60 db. A Nicolet insert earphone (Nicolet Biomedical Instruments, Madison, WI) was used.

**Histopathology.** After the final BAER recordings, all sheep were euthanized with intravenous barbiturate and the brains removed for gross and microscopic analysis. Brains from two control sheep and 20 of 22 animals fed high S were examined for histopathological changes in the areas of the brain stem in relation to the electrophysiological events recorded on BAER. Brains were fixed in 10% buffered formalin and after fixation were sliced transversely to enable anatomical examination. Slices taken were routinely processed, embedded in paraffin, sectioned at 0.5  $\mu$ m and then stained with haematoxylin and eosin. Lesions were histologically graded; grade 1 = mild to moderate edema with mild gliosis and mild vascular endothelial swelling; grade 2 = severe edema with vacuolation of the neuropil, severe neuronal necrosis, moderate gliosis and severe vascular endothelial swelling; and grade 3 = total necrosis of all neural elements with Gitter cell formation and/or astrocytic scar formation.

**Blood thiamine.** Blood thiamine concentration of all animals was monitored throughout the experiment using the biological method (*Ochromonas danica*) of Olkowski and Gooneratne (1991, see appendix) which is a modification of that of Baker and Frank (20).

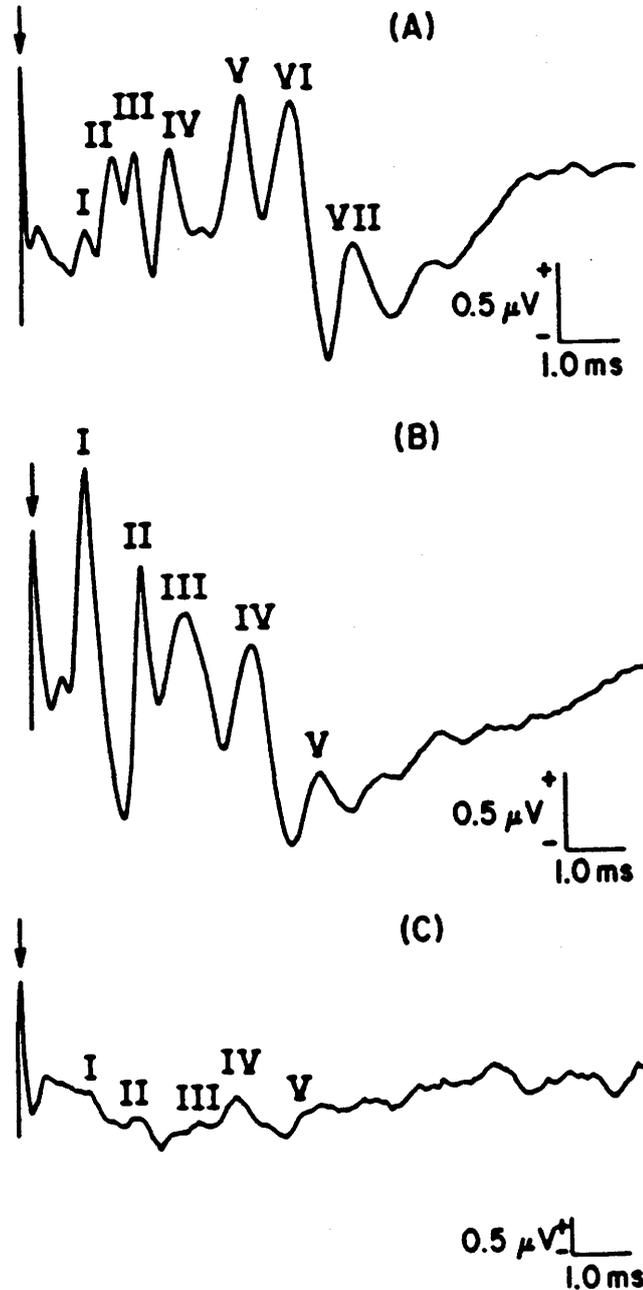
**Statistics.** The General Linear Model Analysis of Variance (GLM ANOVA) from the microcomputer package [Number Cruncher Statistical System (NCSS)] was

used to determine treatment effects. Fisher's LSD (21) test was used for mean comparisons among treatment groups.

## RESULTS

None of the animals tested exhibited characteristic signs of PEM at the time of BAER recording. However three animals fed the high S diet with no supplemental thiamine showed loss of appetite, mild depression and intermittent signs of excitation between 2 and 3 weeks after commencement of the experiment. These signs lasted only for 2 - 3 days and similar signs were not observed during the following 11 weeks prior to the BAER recording at week 14.

The normal pattern of BAER waveforms from a sheep on a low (L) S diet is shown in Fig. 1A. Waveforms, numbering of waves and anatomical relation of peaks were based on those published for horse (13) and human (9) since no data were available for sheep. A tentative scheme of anatomical-electrophysiological relationships was derived assuming a similarity among mammals (22). Anatomical differences such as sizes of nuclei and lengths of the fibers may exist in the brain stem of different species but the electrophysiology is quite similar (22). All animals demonstrated at least 4 major positive peaks and up to 7 could be detected in some sheep (Fig.1A). In sheep given high (H) S diets, abnormal BAER patterns were observed with alterations in amplitude and latency periods, the most marked being in sheep which received either LB<sub>1</sub>-LCu-LMo (Fig.1B) or LB<sub>1</sub>-HCu-LMo (Fig. 1C). Figure 1B shows the presence of all peaks but with apparent alterations in amplitude and latency period.



Figs. 1: (A) The pattern of BAER recording from a control animal fed low Cu-low Mo-low S-low B<sub>1</sub> diet. Several peaks identified as I,II,III,IV and V of differing amplitude are present. (B) The pattern recorded during the 8th week of the trial from an animal (lamb #229) fed high S-low Cu-low Mo-low B<sub>1</sub> diet. Note increment of interpeak latencies. (C) A recording from an animal (lamb #186) fed high S-high Cu-low Mo-low B<sub>1</sub> diet for 14 weeks. A complete disappearance of all waves would indicate lesion at the beginning of the auditory pathway. Each trace is an average of 2000 responses. (↓) recording artifact.

Figure 1C shows a BAER recording with severely depressed amplitude and increased latency. Recordings from three sheep fed diets high in S and low in thiamine (also high in either Mo or Cu) are shown with reference to respective schematic drawing of the damaged areas of the brain in Figs. 2, 3 and 4 respectively. Morphological damage was evident in all animals on this diet and consisted of edema, endothelial swelling of blood vessels, variable neuronal necrosis and gliosis. Fig. 2 is a recording from lamb #23 fed HS-LCu-HMo-LB<sub>1</sub> with mild bilateral lesion in the area of lateral lemniscus. A noticeable decrement in the amplitudes of all BAER components was observed but on one side (right) only, but latencies were increased on both sides (right and left). Fig. 3 depicts the effect of a moderate to severe unilateral lesion of the lateral lemniscus on the BAER recording of sheep #72 which was fed HS-LB<sub>1</sub>-LCu-HMo. The amplitudes on both sides were markedly diminished. Fig. 4 is a recording for sheep #130 (LB<sub>1</sub>-HCu-LMo-HS) showing reduced amplitudes of all components of BAER on both sides (right and left). This sheep had multiple necrotic lesions in the cochlear nuclei region on the right side only. Fig. 5 shows BAER recordings on the 8th and 14th week of the experiment from a sheep fed the HS-LCu-LMo-LB<sub>1</sub> diet and illustrates a progressive change in nerve conduction with time. A mild lesion of the lateral lemniscus region was noted in this animal. The differences in wave patterns indicate the deteriorating effect of high S treatment on brain stem auditory pathway conduction.

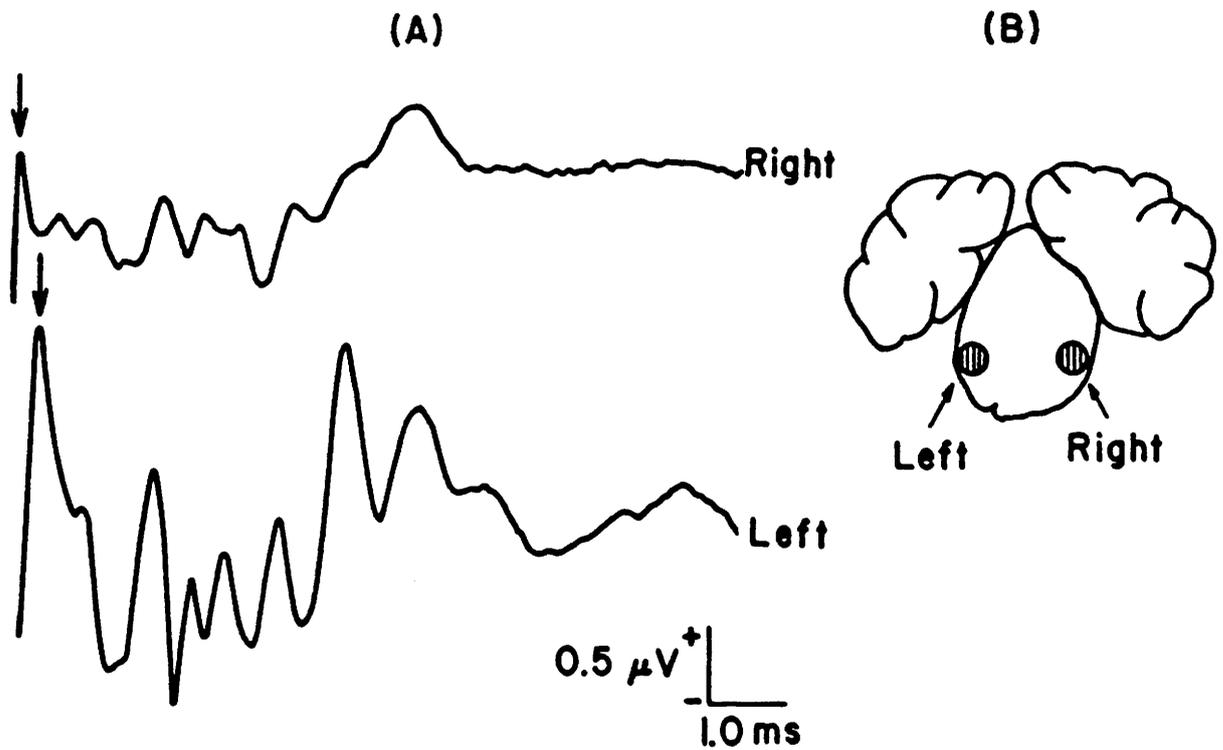


Fig. 2. BAER recording (A) of lamb #23 fed high S-low Cu-high Mo-low  $B_1$  diet. ( $\downarrow$ ) recording artifact. At post mortem a mild bilateral lesion (grade 1;  $\textcircled{\text{||||}}$ ) in lateral lemniscus was seen on both sides (B).

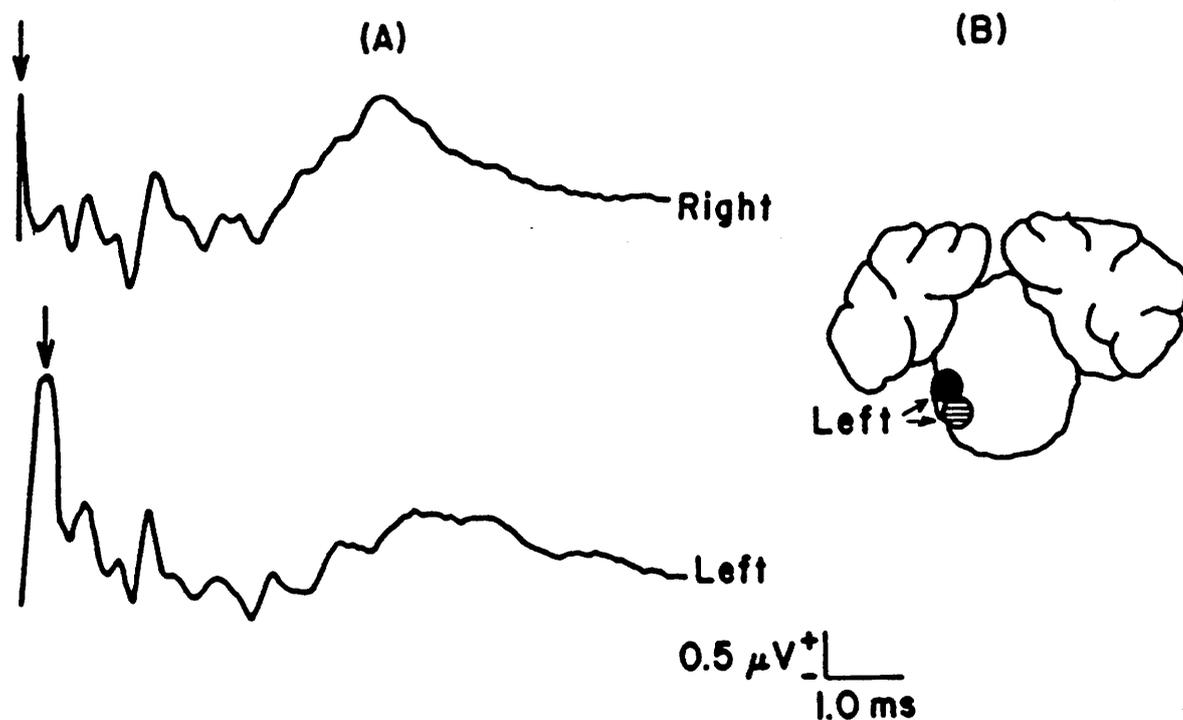


Fig. 3. BAER recording (A) of lamb #72 fed high S-low Cu-high Mo-low B<sub>1</sub> diet. (↓) recording artifact. At post mortem a severe (grade 3; ●) to moderate (grade 2; ⊖) unilateral lesion of lateral lemniscus was seen (B). Note significant decrement of amplitudes in recording in left and right ear.

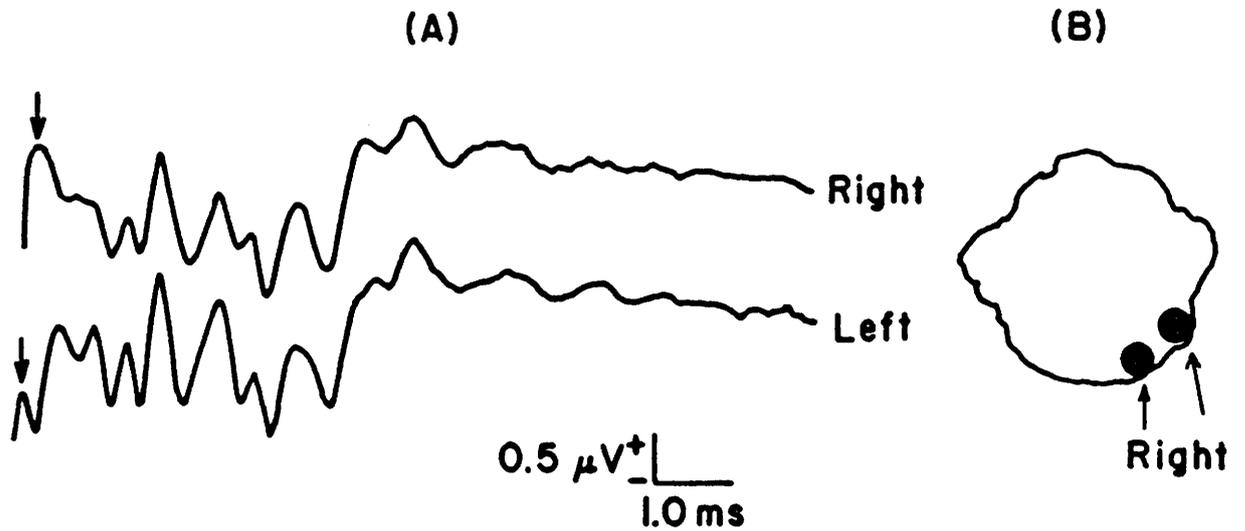


Fig. 4. BAER recording (A) of lamb #130 fed high S-high Cu-low Mo-low B<sub>1</sub> diet. (↓) recording artifact. At post mortem severe multiple lesions (grade 3; ●) in right cochlear nuclei region were seen (B).

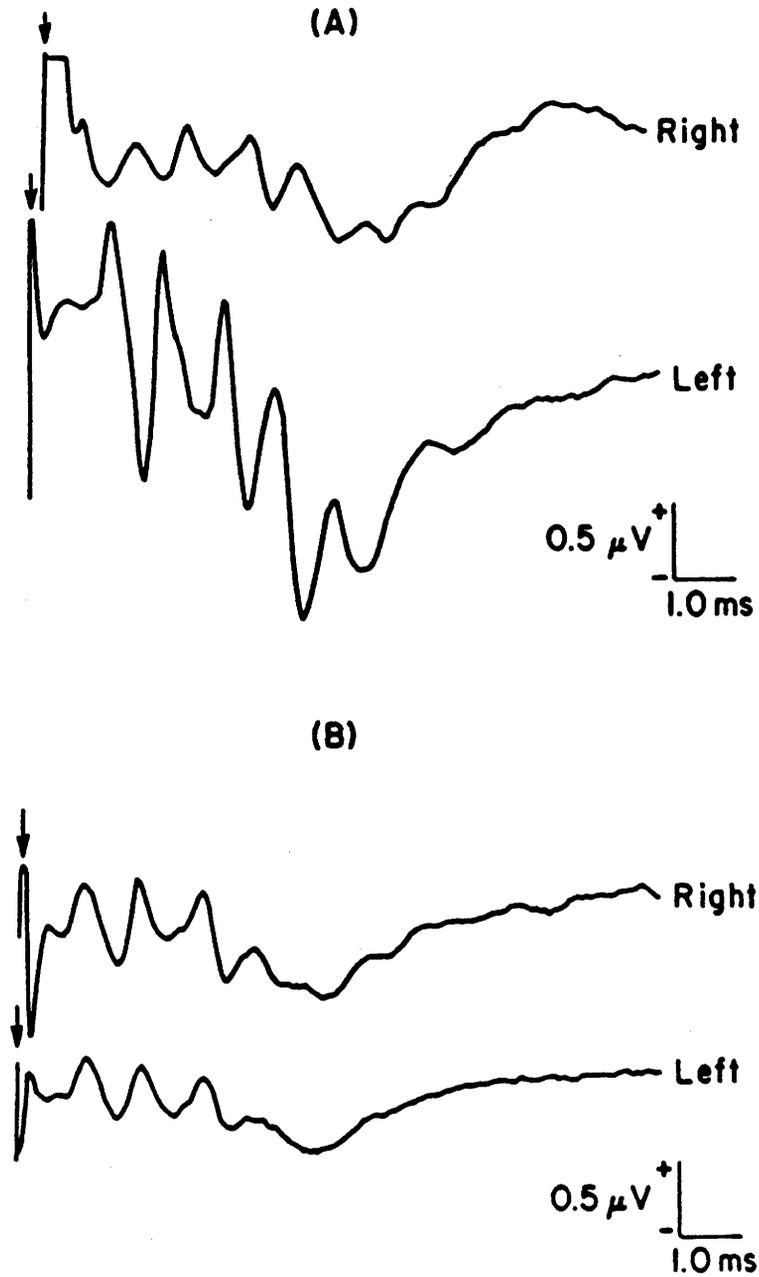


Fig. 5. A comparison of BAER recordings obtained from an animal fed high S-low Cu-low Mo-low  $B_1$  diet on the 8th (A) and 14th (B) week. Note changes on the left side with of feeding excess S over time. (↓) recording artifact. A mild lesion of lateral lemniscus was seen at post-mortem in this animal.

Interpeak latencies (between peak I and IV) of BAER recording of sheep in different treatment groups are shown in Table I. Significantly increased latencies ( $p < 0.05$ ) indicative of reduced nerve conduction were seen in sheep fed the high S diets not supplemented with thiamine (Groups 1, 2, 3, 4) in comparison to those receiving thiamine. The most affected were the sheep on diets 1 and 4 (Table I). The sheep from these two groups had greater latency periods than those on corresponding thiamine supplemented diets 1A and 4A. The latencies of sheep in the remaining two groups which received supplemental thiamine (diet 2A and 3A) were not different to the latencies observed in sheep in corresponding groups not supplemented with thiamine (diets 2 and 3). Factorial analysis revealed the effect of supplemental dietary thiamine to be highly significant ( $P < 0.0001$ ) in lowering the interpeak latency period regardless of the level of Cu or Mo in the diet. The interaction among Cu, Mo, and thiamine was also significant.

**Histopathology.** Most animals fed a high S diet developed lesions of PEM, with variable degrees of severity in the telencephalon, diencephalon, mesencephalon and metencephalon. No difference was noted in grade of lesions among sheep supplemented with thiamine, except in the extra-pyramidal system. Two out of eight animals which received thiamine supplementation had mild brain lesions (grade 1) in the cochlear nuclei or the lateral lemniscus region. Among the 12 sheep which did not receive supplemental thiamine, two animals had grade 3 lesions and three animals had grade 1 lesions and grade 2 lesions were found in the remainder in the above areas of the brain stem.

TABLE I. Interpeak latencies (between I and IV) of Brain Stem Auditory Evoked Response (BAER) Recordings from Sheep Fed Diets Containing High sulfur (0.63%) with Varied Levels of Copper (Cu), Molybdenum (Mo) and Thiamine (B<sub>1</sub>). Control Sheep Were Fed Low Levels of S, Cu, Mo and High B<sub>1</sub>.

Diet No:	Thiamine Supplementation <sup>a</sup>	Diet description <sup>b</sup>	(n)	Interpeak latency mean ± SE (msec)
Control	+	LS-LCu-LMo-HB <sub>1</sub>	4	1.49 ± 0.08 <sup>e</sup>
1	-	HS-LCu-LMo-LB <sub>1</sub>	6	2.69 ± 0.16 <sup>c</sup>
2	-	HS-LCu-HMo-LB <sub>1</sub>	8	2.26 ± 0.14 <sup>cd</sup>
3	-	HS-HCu-LMo-LB <sub>1</sub>	8	2.29 ± 0.14 <sup>cd</sup>
4	-	HS-HCu-HMo-LB <sub>1</sub>	2	2.75 ± 0.28 <sup>c</sup>
1A	+	HS-LCu-LMo-HB <sub>1</sub>	2	1.43 ± 0.28 <sup>e</sup>
2A	+	HS-LCu-HMo-HB <sub>1</sub>	6	1.88 ± 0.16 <sup>de</sup>
3A	+	HS-HCu-LMo-HB <sub>1</sub>	6	1.75 ± 0.16 <sup>d</sup>
4A	+	HS-HCu-HMo-HB <sub>1</sub>	6	1.96 ± 0.16 <sup>de</sup>

ANOVA: Factorial analysis of dietary components.

	Source of variation						
	B <sub>1</sub>	Cu	Mo	B <sub>1</sub> ×Cu	B <sub>1</sub> ×Mo	Cu×Mo	B <sub>1</sub> ×Cu×Mo
P<	0.0001	NS	NS	NS	NS	NS	0.046

<sup>a</sup> Sheep supplemented with thiamine (+) received 243±22.2 mg B<sub>1</sub> Kg<sup>-1</sup> DM diet (HB<sub>1</sub>) compared with a concentration of 13.7±0.4 mg Kg<sup>-1</sup> (LB<sub>1</sub>) received by non-supplemented group (-)

<sup>b</sup> Diet description: Concentration of variable ingredients in the diets expressed as mean ± SD mg Kg<sup>-1</sup> DM.

Low copper (LCu)=6.7±1.1, High copper (HCu)=28.8±1.1, Low molybdenum (LMo)=6.2±1.8, High molybdenum (HMo)=22.5±2.7, Low sulfur (LS)=0.19% S DM, High sulfur (HS)=0.63% DM.

n - refers to number of BAER recordings performed per treatment.

<sup>cde</sup> values with different superscripts within the column differed (p<0.05)

**Blood thiamine.** Blood thiamine concentration of control and test animals are shown in Fig. 6. The concentration of thiamine in control sheep and the test sheep which received thiamine supplementation (Gp 1A, 2A, 3A, 4A) were significantly higher ( $p < 0.0001$ ) than in those test sheep which did not receive thiamine supplementation.

## DISCUSSION

This study showed altered nerve conduction in the auditory pathway in sheep fed high S diets without supplemental thiamine compared with animals which received additional thiamine in the diet. This change in conduction was seen to co-exist with morphological changes along the neuronal tracts providing dual evidence for central nervous system (CNS) damage following S supplementation. Alterations to BAER recordings have rarely been related to histopathologic lesions in domestic animals. The principal changes of BAER components observed in sheep given high S thiamine-unsupplemented diets were 1) increases in interwave interval, 2) increases or decreases in amplitude, and 3) elimination of specific peak components.

Brain stem lesions have been shown in experimentally induced thiamine deficiency in dogs (23). Although sheep which did not receive supplemental thiamine were significantly more affected in terms of BAER recordings, lesions in pertinent brain stem regions were not observed in all examined animals, indicating that BAER may be a more sensitive method of evaluating CNS damage in sheep. Changes in BAER pattern have been related to lesions in specific areas

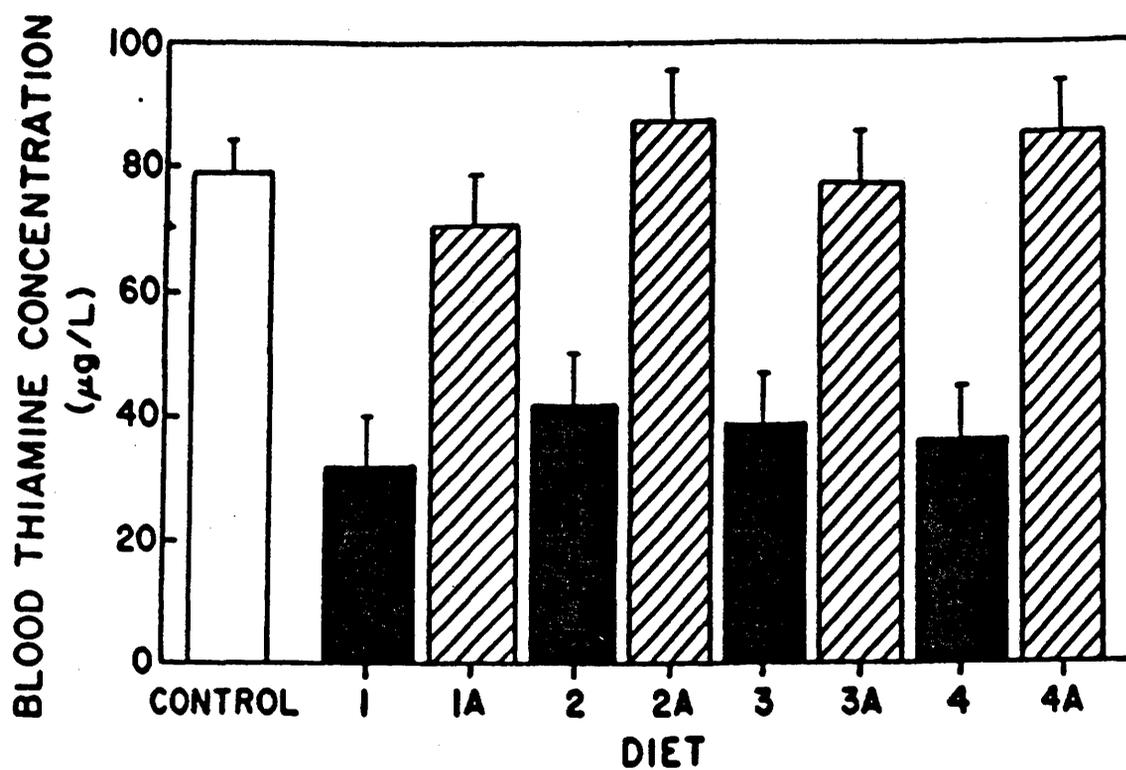


Fig. 6. Blood thiamine concentration ( $\mu\text{g L}^{-1}$ ; Mean  $\pm$  SE) of 2 control sheep fed low-S low-Cu low-Mo high-  $B_1$  diet (  $\square$  ) and 22 test sheep fed high S diet with (  $\text{hatched}$  ) (diet 1A, 2A, 3A, 4A;  $n=10$ ) or without (  $\blacksquare$  ) (diet 1, 2, 3, 4;  $n=12$ )  $B_1$  supplementation. For diet description (see Table I).

of the brain in humans (9,12,24), cats (25) and dogs (26). Although interpretation of such data in establishing pathogenesis of nervous disease is not yet clear it does indicate the possible use of BAER for ante-mortem diagnosis of CNS disease.

It is noteworthy that none of the animals tested exhibited characteristic symptoms of PEM at the time of BAER recording although clinical signs were noted in some animals a few weeks before the readings were made. Therefore, changes in BAER waveforms may indicate a subclinical form of the disease at the time of recording. Changes observed in recordings taken from the same animal at week 8 and 14 (Fig. 5) probably indicates the progressive development of brain lesion with time. Such serial recording and its correlation to a pathologic lesion, although it may not explain the mechanism of an abnormal BAER, may prove to be extremely useful in clinically assessing the progress of a patient.

It is not known whether lesions were present at the time of BAER recording of sheep #229 during the 8th week of the experiment, but we speculate that changes in BAER may also occur in the absence of gross or histological lesions when an ultrastructural or a "metabolic lesion" is present. The term metabolic lesion applies to an impairment of a vital enzyme or a coenzyme or the synthesis or release of neurotransmitter(s). It may be due to the toxic effects S, S metabolites or S mediated B1 deficiency. The cases of PEM in this study and those previously reported associated with high S intakes (2,3) are now presumed to be caused by a complex metabolic interaction of S with other minerals and vitamins (5).

Thiamine metabolism in the brain is unique because it appears to have regionally different requirements for thiamine (6). Chronic thiamine depletion has been associated with selective focal necrosis of the CNS (27). Thiamine deficiency may affect brain metabolism, by altering glucose and energy metabolism, and this may lead to a compromise of a variety of metabolic systems in the CNS. Myelin production can be impaired (6), and alterations in concentration of brain neurotransmitters has been reported (28,29,30). Thiamine is an integral part of nerve membranes (31,32), and hence plays a major role in nerve excitation and conduction. Since it is now known that high dietary S can cause thiamine depletion in ruminants (5), the changes in brain metabolism described above may possibly have resulted in prolonged interpeak latencies observed in the BAER recordings of the present study.

In conclusion, the evidence from this experiment indicates that high dietary S-related thiamine deficiency is one of the key factors associated with abnormal BAER, although interactive effects of Cu and Mo with thiamine were also significant in inducing changes to latencies of BAER recordings.

#### **ACKNOWLEDGMENTS**

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**SULFUR-INDUCED POLIOENCEPHALOMALACIA IN SHEEP:  
SOME BIOCHEMICAL CHANGES**

S.R. Gooneratne, A.A. Olkowski and D.A. Christensen. *Can. J. Vet. Res.* 1989, Vol.53:462-467.

**ABSTRACT**

The effect of high dietary sulfur (S) supplementation on blood thiamine (B<sub>1</sub>) concentration, biochemical indices of liver, muscle and kidney damage and selected plasma electrolytes was studied in six sheep. Three of these sheep received an additional 230 mg thiamine/kg diet (Group 2). After approximately 2.5-3 weeks on this diet, all three sheep in the non-B<sub>1</sub>-supplemented group (Group 1) showed loss of appetite and developed mild neurological signs: depression, intermittent signs of excitation and head pressing. Increases in blood B<sub>1</sub> concentration and plasma creatine kinase (CK) and aspartate amino-transferase (AST) were observed during this time in all affected animals. Clinical signs lasted only for 2-5 days. Sheep in Group 2 were clinically normal throughout the experiment, but all of these animals also had elevated blood B<sub>1</sub> concentrations and plasma CK activity at the 3 wk sampling. Plasma magnesium concentrations of Group 1 sheep were elevated at the 2.5-3 wk and 6 wk samplings but they declined significantly ( $p < 0.05$ ) to low normal levels thereafter. Magnesium concentrations of Group 2 sheep were low at the beginning but progressively increased during the course of the experiment. At necropsy, brain lesions suggestive of polioencephalomalacia (PEM) were observed in all sheep but were

most marked in Group 1. It is speculated that PEM may be caused by a direct toxic effect of S, S metabolites or B<sub>1</sub> antimetabolites in the brain rather than by an in vivo B<sub>1</sub> deficiency per se.

## INTRODUCTION

Polioencephalomalacia (PEM) is a neurologic disease of ruminants. It is commonly associated with feeding a high level of concentrate and/or a low amount of roughage (1). Recent studies have shown that diets (2) or drinking water (3,4) containing high levels of sulfates also predispose cattle to PEM, but little is known of the mechanisms or the biochemical changes associated with S-induced PEM in ruminants. Although biochemical findings on experimental and field cases of PEM are consistent with lowering of thiamine (B<sub>1</sub>) status, there is no complete agreement in the literature that the blood B<sub>1</sub> levels are always reduced in affected animals. Studies on PEM have shown that blood B<sub>1</sub> levels may be decreased (5) while other reports have shown them to be within normal limits (6,7) or even elevated (8). The reason for such discrepancy is not clear but if PEM is induced by analogues such as amprolium or by B<sub>1</sub> antimetabolites blood B<sub>1</sub> concentrations may not always be low.

Magnesium (Mg) is a co-factor of several B<sub>1</sub>-containing enzymes (9). Metabolic interactions between minerals, and between minerals and other components in the diet and body fluids are well recognized (10). Changes in plasma concentration of the electrolytes sodium (Na), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and chloride (Cl) have been reported in

naturally occurring PEM (11) and in amprolium-induced B<sub>1</sub> deficiency in sheep (6) and horses (12). Such information is lacking in S-induced PEM in ruminants. Excess S in the diet has been shown to induce muscular dystrophy in ruminants by increasing excretion of selenium (Se) in urine (13). Recent studies have shown an association between B<sub>1</sub> deficiency and hepatorenal syndrome in humans (14). The objective of the present study was to induce PEM experimentally in sheep by feeding high S and to examine changes in blood B<sub>1</sub> concentration, certain plasma electrolyte concentrations and biochemical indices of liver, muscle and kidney damage during the progression of the disease.

## **MATERIALS AND METHODS**

**Animals, diets and biochemical analyses.** Six, 8-week old lambs of nondescript breed of mean body weight 12 - 15 kg were used. All lambs were fed a barley-soybean meal-alfalfa based diet containing either high (H) S (0.63%) - basal (L) B<sub>1</sub> (13.7 mg/kg DM) (Group 1, n=3) or HS-HB<sub>1</sub> (230 mg/kg DM of B<sub>1</sub>) (Group 2, n=3). Blood B<sub>1</sub> was measured using the biological method (*Ochromonas danica*) of Olkowski and Gooneratne [(15), see appendix]. Measurements of plasma concentrations of creatine kinase (CK) (16), aspartate amino-transferase (AST) (17), gamma-glutamyl transferase (GGT) (18), creatinine (CRT) (19), urea (20), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), magnesium (Mg), were carried out using a Discrete Analyzer with Continuous Optical Scanning (DACOS, Coulter Electronics Inc. Hialeah, Florida) prior to initiation of the trial, at every 3 wk period thereafter, and on days when an animal showed neurological

symptoms until the termination of the experiment. Since these animals were a part of a bile collection experiment, after 8 wks on the diets, ligation of the bile duct, and cannulation of the gall bladder and mid-third of duodenum were performed on all sheep as described by Caple and Heath (21) except sheep #81 in Group 2. Clinical appearance and vital neurological signs were monitored daily. The experiments followed the guidelines of the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

**Statistics.** Statistical analysis was carried out using General Linear Model analysis of Variance (GLM ANOVA) from microcomputer package [Number Cruncher Statistical System (NCSS)]. Fisher LSD (22) test was used to determine statistical significance of means between the treatment groups.

## RESULTS

**Clinical signs.** All animals in Group 2 which received supplemental B<sub>1</sub> appeared clinically normal throughout the experiment. In contrast, all three sheep fed the HS-LB<sub>1</sub> diet (Group 1) showed loss of appetite and mild neurological signs such as mild depression, intermittent signs of excitement and head pressing within 2-3 wk. Two sheep recovered completely within 2-5 days but sheep #47 died suddenly on day 37 of the experiment. No signs of illness were observed in the other two animals in the group during the remainder of the experiment. Necropsy and/or histological findings indicative of PEM, such as cortical and mid-brain focal necrosis (23) were seen in all sheep, but the lesions were most

severe in sheep #47 and in the remaining Group 1 sheep (Olkowski et al, unpublished results).

**Blood thiamine and plasma enzyme changes.** Blood B<sub>1</sub> concentrations of all lambs were within the reference limits (5) prior to the start of the experiment (Table I) but two sheep (#47 and #72) in Group 1 which exhibited neurological signs had increased levels of up to 242 and 173 g/L at the height of signs (2-3 wk). Aspartate aminotransferase activity was increased in all lambs in Group 1 at this time but CK activity was increased only in two lambs. These changes were most marked in sheep #47 (Fig. 1). Samples taken at 3 wk from apparently clinically normal sheep in Group 2 also showed markedly elevated blood B<sub>1</sub> concentration (Table I). Plasma CK was elevated in two of three lambs in this group. These changes were most marked in lamb #78 (Fig. 1). Plasma AST activity was also markedly elevated in this lamb during this time. Plasma GGT activity remained within normal limits in all six animals during the initial 6 wk period.

Blood B<sub>1</sub> concentration of lambs in both groups returned to within normal limits after 3 wk, but plasma CK was elevated even at 6 wk. Both CK and AST activities fluctuated during the remainder of the experiment. From 9 wk onwards GGT activities were increased in all sheep except lamb #81 (Group 2) which was not cannulated. Plasma creatinine and urea levels of all animals remained within the normal limits of 69-105  $\mu\text{mol/L}$  and 0-10 mmol/L respectively.

TABLE I - Changes in Blood Thiamine (B<sub>1</sub>) Concentration, and Plasma Activities of Creatine Kinase (CK), Aspartate Amino Transferase (AST) and gamma-glutamyl Transferase (GGT) in Sheep Fed High Sulfur With (Group 2) or Without (Group 1) Supplemental B<sub>1</sub> in the Diet.

	<u>Group 1<sup>ab</sup></u>					<u>Group 2<sup>b</sup></u>				
	<u>High S - Basal B<sub>1</sub> Diet</u>					<u>High S - High B<sub>1</sub> Diet</u>				
	<u>Time of Sampling (wk)</u>					<u>Time of Sampling (wk)</u>				
	0	2.5-3	6	9	12	0	3	6	9	12
Blood B <sub>1</sub> concentration (µg/L)	51 <sup>d</sup> ±31	207 <sup>c</sup> ±28	73 <sup>d</sup> ±4	50 <sup>d</sup> ±1	44 <sup>d</sup> ±11	57 <sup>d</sup> ±4	742 <sup>c</sup> ±284	97 <sup>d</sup> ±4	89 <sup>d</sup> ±3	90 <sup>d</sup> ±2
Plasma CK <sup>e</sup> activity (U/L)	312 <sup>cd</sup> ±109	513 <sup>c</sup> ±145	386 <sup>cd</sup> ±104	276 <sup>cd</sup> ±162	118 <sup>d</sup> ±12	245 ±14	750 ±482	654 ±333	93 ±39	280 ±137
Plasma AST <sup>e</sup> activity (U/L)	91 <sup>d</sup> ±1	112 <sup>d</sup> ±11	91 <sup>d</sup> ±4	300 <sup>c</sup> ±166	149 <sup>cd</sup> ±11	106 ±21	324 ±240	144 ±9	73 ±22	287 ±126
Plasma GGT <sup>e</sup> activity (U/L)	73 <sup>d</sup> ±5	63 <sup>d</sup> ±6	61 <sup>d</sup> ±4	449 <sup>c</sup> ±4	470 <sup>c</sup> ±44	68 <sup>d</sup> ±2	59 <sup>d</sup> ±4	60 <sup>d</sup> ±5	159 <sup>d</sup> ±8	385 <sup>c</sup> ±43

<sup>a</sup> Number of animals sampled from each group was three except at 6, 9 and 12 wk in Group 1 when only two animals were sampled.

<sup>b</sup> Results are expressed as mean ± SD.

<sup>cd</sup> Means within a row and within treatment followed by different letter superscripts are significantly different (P<0.05).

<sup>e</sup> Reference values for enzymes CK, AST and GGT set by the Clinical Pathology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan are <350, 48 to 128 and <70 U/L respectively.

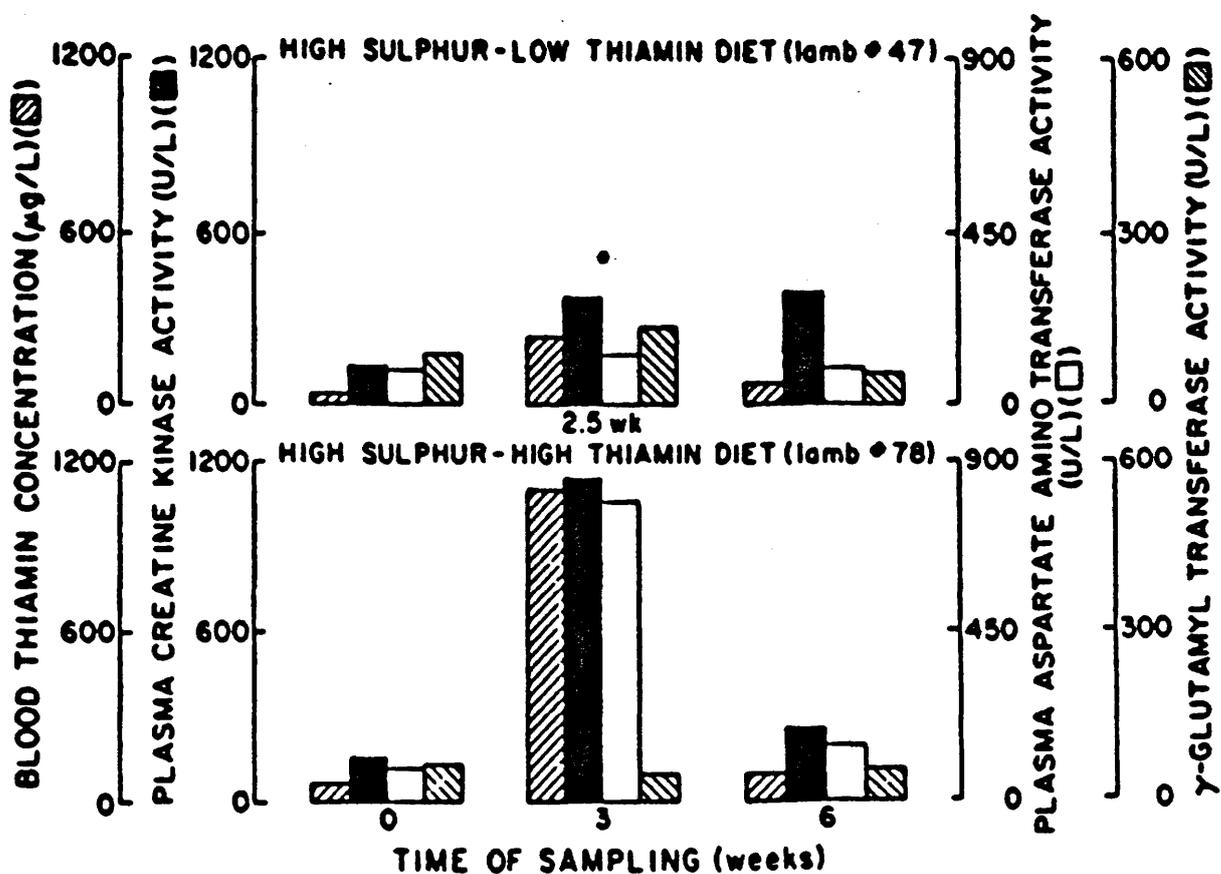


Fig. 1. Changes in blood thiamine concentrations and activities of creatine kinase, aspartate aminotransferase and gamma-glutamyl transferase in plasma of a sheep given high sulfur-low thiamine diet (sheep #47) and one given high sulfur-high thiamine diet (sheep #78). The asterisk (\*) indicates the time when neurological signs were observed in sheep #47.

The blood B<sub>1</sub> concentration of a sample from sheep #47 in Group 1, taken 24-48 h prior to its death was within the normal range, but plasma CK activity was elevated (588 U/L). Both AST and GGT activities of this animal were within normal limits at this time.

**Plasma electrolytes.** Changes in plasma concentrations of Na, K, Cl, Mg, Ca and P of sheep in both groups during the course of the study are given in Table II. Potassium concentrations remained within reference limits (4.6 - 7.0 mmol/L) in all lambs in Group 1 but all lambs in Group 2 exhibited hyperkalemia (up to 10 mmol/L) during week 3. This persisted in two of three animals until 6 wk but decreased thereafter. Changes in Na were minor but Cl concentrations declined significantly to below normal level at 9 wk in all Group 2 sheep.

Among Group 1 sheep, plasma Mg concentrations increased to above normal levels during the 2-3 and 6 wk samplings but declined significantly ( $P < 0.05$ ) to low marginal levels thereafter. On the other hand, Mg concentrations of sheep in Group 2 were low at the beginning of the experiment (normal range = 0.90 - 1.26 mmol/L) but increased during the course of the experiment. Slightly above normal levels were detected at the 9 wk sampling.

Plasma Ca concentrations remained within normal limits in all sheep throughout the course of the experiment. Plasma P concentrations were above normal (normal range 0.82 - 2.66) in all animals at the onset of the experiment. Increases in plasma P were observed in both groups during the initial 6 wk.

TABLE II - Plasma Electrolyte Concentration<sup>a</sup> of Lambs Fed High Sulfur (S)-Normal Thiamine (B<sub>1</sub>) (Gr. 1) and High S-High B<sub>1</sub> Diets (Gr. 2).

<u>Electrolyte</u> (mmol/L)	<u>Group 1<sup>a</sup></u>					<u>Group 2<sup>a</sup></u>				
	<u>High S - Basal B<sub>1</sub> Diet</u>					<u>High S - High B<sub>1</sub> Diet</u>				
	<u>Time of Sampling (wk)</u>					<u>Time of Sampling (wk)</u>				
	0	2.5-3	6	9	12	0	3	6	9	12
Sodium <sup>d</sup>	149 ±2	148 ±2	147 ±1	148 ±3	147 ±1	149 ±3	146 ±2	143 ±3	143 ±4	149 ±1
Potassium <sup>d</sup>	5.6 <sup>bc</sup> ±0.6	5.6 <sup>b</sup> ±0.1	6.1 <sup>b</sup> ±0.3	4.7 <sup>c</sup> ±0.1	4.6 <sup>c</sup> ±0.3	5.7 <sup>bc</sup> ±0.4	7.7 <sup>b</sup> ±1.3	8.1 <sup>b</sup> ±1.2	4.7 <sup>c</sup> ±0.7	5.1 <sup>bc</sup> ±0.5
Chloride <sup>d</sup>	107 ±2	107 ±2	107 ±1	105 ±1	109 ±1	106 <sup>b</sup> ±1	107 <sup>b</sup> ±1	105 <sup>b</sup> ±2	89 <sup>c</sup> ±11	104 <sup>b</sup> ±1
Magnesium <sup>d</sup>	1.0 <sup>bc</sup> ±0.1	1.3 <sup>b</sup> ±0.1	1.3 <sup>b</sup> ±0.2	0.9 <sup>c</sup> ±0.1	0.9 <sup>c</sup> ±0.1	0.8 <sup>c</sup> ±0.1	1.0 <sup>bc</sup> ±0.1	1.1 <sup>bc</sup> ±0.1	1.3 <sup>b</sup> ±0.3	1.1 <sup>bc</sup> ±0.1
Calcium <sup>d</sup>	2.8 ±0.1	2.6 ±0.1	2.6 ±0.2	2.7 ±0.1	2.7 ±0.1	2.8 ±0.1	2.6 ±0.1	2.5 ±0.1	2.4 ±0.1	2.6 ±0.1
Phosphorus <sup>d</sup>	2.8 <sup>c</sup> ±0.4	3.2 <sup>bc</sup> ±0.2	3.6 <sup>b</sup> ±0.3	2.8 <sup>c</sup> ±0.1	2.6 <sup>c</sup> ±0.2	2.9 <sup>c</sup> ±0.3	3.1 <sup>b,c</sup> ±0.3	3.1 <sup>bc</sup> ±0.4	3.7 <sup>b</sup> ±0.6	3.7 <sup>b</sup> ±0.3

<sup>a</sup> Results are expressed as mean ±SD. Number of animals sampled from each group was three except at 6, 9 and 12 wk in Group 1 when only two animals were sampled. Sheep #47 from this group died on day 37 of the experiment.

<sup>bc</sup> Values with different letter superscripts within a row and within treatment differ significantly (P<0.05).

<sup>d</sup> Reference values for electrolytes sodium, potassium, chloride, magnesium, calcium and phosphorus as recognized by the Clinical Pathology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan are 143 to 151, 4.6 to 7.0, 102 to 116, 0.9 to 1.26, 2.3 to 2.86 and 0.82 to 2.66 mmol/L respectively.

The levels of P in Group 1 sheep declined thereafter but the plasma P concentration of lambs in Group 2 continued to increase throughout the experiment and high levels were observed at the termination of the study.

## DISCUSSION

The present study has shown that PEM can be experimentally produced in sheep given high levels of S in the diet. This confirms observations recorded from field investigations that PEM can be caused by consumption of either high S (as sulfate) in the ration (2) or in the water (3,4,24). This is also consistent with the finding that in vitro sulphite is capable of cleaving B<sub>1</sub> in aqueous solutions (25). However Edwin et al (26) were unable to produce PEM in young sheep by feeding 15 g of sodium sulfite for up to one year. In the present study excess S was fed only as sulfates and it is not known whether the ionic configuration of this form of S had an influence on the development of PEM. In the present study, neurological signs indicative of B<sub>1</sub> deficiency were not observed in B<sub>1</sub>-supplemented sheep. However, brain lesions suggestive of PEM (Olkowski et al, unpublished results), were seen in all three sheep supplemented with B<sub>1</sub> but the lesions were far less severe (23). This suggests that factors other than deficiency of B<sub>1</sub> may be responsible for the development of brain pathology of sheep fed high S diets.

Increases in blood B<sub>1</sub> concentrations during the appearance of early neurologic symptoms of PEM have not been reported previously. However above normal blood B<sub>1</sub> levels have been reported in clinically normal animals within 1-2

wk of feeding amprolium, a B<sub>1</sub> analogue, to calves (8). This author used the thiochrome method for estimation of blood B<sub>1</sub> concentrations and the possibility that amprolium in blood may have interfered with the assay (7) cannot be ruled out. Slight elevations of blood B<sub>1</sub> accompanied by corresponding increases in urine B<sub>1</sub> have also been reported in sheep within 2-3 wk of feeding a bracken rhizome diet (27). In the latter study a second elevation of both blood and urine B<sub>1</sub> levels was observed after 4-5 wk of feeding bracken. Nervous signs were not detected on either occasion. Increased excretion of B<sub>1</sub> in urine has been reported in calves given a high carbohydrate diet (28), or amprolium (8). We have observed (Olkowski et al, unpublished observations) similar increases in urine B<sub>1</sub> in all sheep fed high S. As expected such increases were most marked in sheep supplemented with B<sub>1</sub>.

The relevance of the increase in blood B<sub>1</sub> concentrations during early stages of the disease and its relationship to the appearance of mild neurological signs as observed in the present study is not clear. This is because extremely high blood B<sub>1</sub> concentrations were also observed in apparently normal B<sub>1</sub>-supplemented sheep during the same time. But sheep fed low S diets do not show such elevations in blood B<sub>1</sub> concentration (Olkowski et al, unpublished results). The origin of the elevated B<sub>1</sub> in the blood of sheep fed high S is not known but it is possible that leakage may have occurred from either the muscle and/or the liver since both plasma CK and AST activities were elevated. Hepatic lesions have been inferred from clinical pathology data of amprolium-induced PEM in horses

(12), but it is unlikely that such marked liver damage occurred in sheep in the present study since normal GGT levels occurred during periods of CK and AST elevations. Elevated levels of both AST and GGT were observed in subsequent weeks: 9 wk and 12 wk in Group 1 sheep and 9 wk in Group 2 sheep. This probably occurred from a mild cholestasis as a result of the indwelling gall bladder catheter in these animals since such an elevation was not observed in the uncannulated sheep #81 in Group 2.

Although muscle does not store  $B_1$  to any great extent, a marked elevation in plasma  $B_1$  due to leakage from muscle is still a possibility because of the large muscle mass in the body. Increases in plasma CK have been previously reported in amprolium-induced PEM in calves (8) but only during periods of violent clonic spasms. Blood  $B_1$  concentrations of such animals were only slightly elevated. But the possibility that blood  $B_1$  concentrations may have peaked in these animals prior to measurement of CK cannot be discounted. Evidence of cardiac lesions, and associated functional cardiac impairment have been reported in  $B_1$  deficiency in the cat (29) and in ruminants (30). The technique used to determine CK in the present study did not differentiate whether its origin was solely from skeletal or cardiac muscle or a combination of both. Creatine kinase is a cytosolic enzyme in muscle. High plasma CK values are indicative of either an increase in muscle cell permeability or acute damage to muscle. Increases in plasma CK have been reported in a variety of muscular disorders including Se deficiency-associated nutritional muscular dystrophy (NMD) in calves (31) and NMD in lambs (32,33).

It is not known whether feeding of a high S diet to sheep precipitates an early Se deficiency but muscular dystrophy has been reported in sheep fed high S diets (13).

Increased plasma CK as observed in the present study does not exclude the possibility of nervous damage being the cause of CK release since elevations of CK have also been reported to occur in a variety of diseases of the nervous system in humans (34) and animals (35). Typical histological lesions of PEM were observed in the brains of all sheep but the lesions were most severe in the animals not supplemented with B<sub>1</sub> (23). It has been postulated that in encephalomalacia, plasma CK of brain origin is not elevated since the blood brain barrier (BBB) is not altered. But starvation and stress, common occurrences during the height of nervous signs, significantly disrupt BBB (36). Dubo et al (34) and Smith and Healy (35) attributed increased CK observed in nervous disorders to abnormal muscle contractions which are a part of the neurological syndrome. Although this may have occurred in Group 1 lambs which exhibited mild neurological symptoms, it is unlikely to be the explanation for the rise in CK observed in Group 2 lambs since neither neurological signs nor an increase in muscular activity were observed in these sheep during the time of CK rise.

Creatine kinase is eliminated rapidly from blood (37) and the half-life of CK derived from sheep is only 62 min (32). Therefore the presence of high CK levels observed in the present study at 3 and 6 wk suggest insult to muscle during these times although more frequent sampling would have confirmed whether such

damage was persistent or not. However the blood  $B_1$  concentrations of all lambs at 6 wk were normal. If blood  $B_1$  originated from muscle, the reason for the return of blood  $B_1$  to normal levels in spite of continued high CK levels is not known. It should be noted that most of the  $B_1$  is associated with cellular mitochondria and only the amount of  $B_1$  present in cytosol would leak out during permeability change or muscle damage. It can be speculated that the initial increase in muscle permeability may have resulted in leakage of almost all of the muscle cytosolic  $B_1$  into the bloodstream leaving little or no  $B_1$  for leakage during subsequent changes in muscle permeability. The marked increase in blood  $B_1$  concentration observed in  $B_1$ -supplemented sheep (Group 2) probably indicates leakage from relatively higher stores of  $B_1$  in muscle and/or liver of these sheep compared to the levels in sheep in Group 1. Since both CK and blood  $B_1$  returned to baseline values after 6 wk it is assumed that the sheep may have recovered from an initial insult caused by high S, or adapted to the high S diet by this time.

Changes in plasma electrolyte concentration observed in the present study are in agreement with previous reports on amprolium-induced PEM in cattle (8, 11). No marked changes were observed in either plasma Na or Ca concentrations. The decline in plasma Mg in Group 1 sheep as opposed to an increase in Group 2 sheep during the terminal stages of the experiment (week 12) is consistent with the finding that the metabolism of  $B_1$  and Mg are interdependent (9). Magnesium is a co-factor of  $B_1$  dependent enzymes, pyruvate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase and transketolase. The reason for the marked increase of plasma

Mg concentration at the 2.5-3 and 6 wk samplings in Group 1 sheep is not clear. Blood  $B_1$  and plasma CK were also elevated in these animals during this time. It has been shown that animals displaying increased muscular activity may have above normal Mg concentration in blood due to a release of Mg from tissues (38). Similar increases in plasma Mg have been recorded in feedlot steers fed an all-concentrate, barley-based diet (39) and 1, 2 and 5 wk following amprolium treatment in cattle (6). The CK was not measured in the latter study. It is unlikely that Mg leaked out from the muscle since Group 2 animals which showed elevated levels of CK and markedly elevated blood  $B_1$  concentration at the 3 wk sampling showed only a marginal increase in plasma Mg.

It is notable that hyperkalemia and elevated blood  $B_1$  occurred concurrently in Group 2 sheep during week 3 and this was also observed at week 6. The highest concentration of plasma K was observed in Group 1 lambs at 6 wk when plasma CK was still marginally high. Increased serum K concentrations have been reported in amprolium-treated geldings during the height of clinical signs (12). Sheep in Group 1 had low-normal plasma K concentrations during the terminal stages of the present study. Low plasma K has been reported in calves during the terminal stages of PEM (40) but not in adult cattle (41). Little is known about P metabolism in animals affected with PEM. The increases in plasma P levels observed in all sheep during the initial 6 wk is probably related to rapid growth and bone turnover in young animals (42). The reason for the decline in plasma P in Group 1 sheep and the increase in Group 2 sheep which

received B<sub>1</sub> supplementation is not known. Decreases in serum P levels have been previously reported in two horses receiving amprolium but these animals in addition also received Mg supplementation (12).

In conclusion, changes in several biochemical parameters observed in high S-related PEM in sheep appear to be similar to those observed in naturally occurring (11) and amprolium-induced PEM in ruminants (6,8,27) and horses (12). It is still debatable whether a localized B<sub>1</sub> deficient state in the brain as suggested by Loew et al (7) is in fact responsible for the appearance of brain lesions and signs of PEM. The present study does not support either a systemic or a localized B<sub>1</sub> deficiency as the cause of high S-related PEM in sheep since both blood B<sub>1</sub> (Table I) and brain B<sub>1</sub> concentrations (2.2-3.0 g/g brain wet weight; Olkowski et al, unpublished results) of all sheep at the conclusion of the experiment were within the normal range [blood: 18-60 g/L (5); brain: 1.29-1.82 g/g wet weight (43)]. Since brain lesions suggestive of PEM were observed in both B<sub>1</sub>-supplemented and unsupplemented groups of sheep, it seems likely that a direct toxic effect of either S, S metabolites, or B<sub>1</sub> antimetabolites on the brain could have been responsible for this occurrence. In view of the abundance of high sulfate water available to livestock in Saskatchewan the concept of a S-induced nervous disorder resembling a B<sub>1</sub> deficiency in ruminants has wide implications of economic importance. Further investigation is needed to establish the mechanisms responsible for the occurrence of this syndrome. Early identification of a subpopulation of animals at risk of development of brain damage in high S-

fed animals would be extremely valuable from the standpoint of clinical management of the problem.

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## THE ROLE OF THIAMINE STATUS IN SULPHUR INDUCED POLIOENCEPHALOMALACIA IN SHEEP

A.A. Olkowski, S.R. Gooneratne, C.G. Rousseaux and D.A. Christensen. Res. Vet. Sci. 1992. Vol. 52:78-85.

### ABSTRACT

The effects of excess dietary sulphur (S) were studied in sheep supplemented and unsupplemented with thiamine. The diets contained either 0.19% S (LS) or 0.63% S (HS) in combinations with 14 mg/kg thiamine (LB<sub>1</sub>) or 243 mg/kg thiamine (HB<sub>1</sub>). A total of 56 two month old lambs were used. Groups consisting of 9, 9, 22, and 16 lambs were fed LS-LB<sub>1</sub>, LS-HB<sub>1</sub>, HS-LB<sub>1</sub>, and HS-HB<sub>1</sub> diets respectively for 14 weeks. Out of 22 lambs fed the HS-LB<sub>1</sub> diet, 7 lambs developed neurological signs between the 3rd and 8th week of the trial. Two of these lambs died, three that were *in extremis* were euthanized, and two recovered completely. All clinically affected animals had extensive malacic lesions in the cerebral cortex, midbrain, and brainstem. None of the lambs from the LS groups or HS-HB<sub>1</sub> group developed clinical signs. Several clinically normal lambs from the HS-LB<sub>1</sub> group had necrotic lesions in their brains at gross and microscopic examination. Supplementation with dietary thiamine prevented development of clinical signs, but did not totally prevent development of microscopic brain lesions. Brain thiamine concentration, transketolase (TK) activity and thiamine pyrophosphate (TPP) effect were not different ( $p>0.05$ ) among groups. There was a strong effect ( $p<0.0001$ ) of dietary thiamine supplementation on blood thiamine concentration

and TPP effect. Blood thiamine concentration was higher whereas TPP effect was lower in the thiamine supplemented sheep. Blood and tissue thiamine concentrations in sheep exposed to high dietary S did not indicate either systemic or local thiamine deficiency per se. Increased TPP effect in sheep fed the HS-LB<sub>1</sub> diet indicates mild-to-moderate metabolic thiamine deficiency. Thiamine inadequacy may be an effect of an increased requirement for thiamine in animals exposed to excess dietary S.

## INTRODUCTION

Polioencephalomalacia (PEM), also known as cerebrocortical necrosis (CCN), was first identified as a distinct pathological entity in ruminants by Jensen et al (1956), and Terlecki and Markson (1961). Although this condition has been recognized for more than three decades, its etiology is still not completely understood. A variety of different factors such as thiamine deficiency (Pill 1967, Daly 1968, Edwin and Jackman 1982), cobalt deficiency (Hartley et al 1962, MacPherson et al 1976), selenium toxicity (Maag et al 1960), molasses toxicity (Mella et al 1976) and lead poisoning (Christian and Tryphonas 1971) have been associated with the development of malacic lesions. More recent reports have pointed to dietary S<sup>a</sup> as a potential cause. The occurrence of clinical signs of PEM has been documented in cattle exposed to a high concentration of sulphate

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<sup>a</sup>Sulphur (S) in this report represents any form (inorganic and organic) of dietary sulphur since in ruminants all forms of dietary S are metabolized to sulphide (S<sup>-2</sup>) and it is not known which ionic form of S causes the effect.

in feed (Raisbeck 1982, Sadler et al 1983) and in water (Harries 1987, Gooneratne et al 1989a). The disease was experimentally induced by feeding excess dietary S to sheep (Gooneratne et al 1989b).

Morphologically, PEM is characterized by a focal necrosis of grey matter in the brain. The lesions are qualitatively similar in all cases of PEM, but it is not clear whether or not PEM has any specific common etiology (Sullivan 1985). However, there is an ample body of evidence that thiamine deficiency or disturbances in thiamine metabolism are implicated in the pathogenesis of this syndrome (Loew 1975, Edwin and Jackman 1982). Thiamine, if administered in early stages of the disease, leads to a prompt and complete clinical recovery (Daly 1968, Thomas 1986, Gooneratne et al 1989a).

The objectives of the present study were to induce PEM by feeding excess S, and to investigate the effect of excess dietary S on blood and tissue thiamine, erythrocyte and tissue TK activity, and to evaluate the effect of thiamine supplementation on the above indices and the development of clinical signs and/or lesions in sheep.

## **MATERIALS AND METHODS**

**Animals and Diets.** Fifty-six cross-bred female sheep approximately 2 months old at the start of the experiment were used. The sheep were housed individually in metabolic crates. The basal diet consisting of barley (59%), soybean meal (5%), alfalfa (32%), and minerals and vitamins mix blended with barley (4%) was prepared to meet recommended nutritional requirements (NAS-NRC 1985). The

basal diet contained 0.19% S and 14 mg thiamine/kg DM. Thiamine (as thiamine HCl) and S (as K,Mg sulphate) were variable ingredients added to the basal diet. All diets were pelleted.

The animals were randomly assigned to four treatments, designed in a 2 X 2 factorial arrangement with low (LS) and high (HS) levels of S, and low (LB<sub>1</sub>) and high (HB<sub>1</sub>) levels of thiamine. Hence, nine control sheep received a basal diet (LS-LB<sub>1</sub>). Nine other sheep were fed a diet containing 243 mg thiamine/kg DM (LS-HB<sub>1</sub>). Twenty-two sheep were fed a diet containing 0.63% S unsupplemented with thiamine (HS-LB<sub>1</sub>). Another sixteen sheep were fed a diet containing 0.63% S and 243 mg thiamine/kg DM (HS-HB<sub>1</sub>). All animals were fed a ration of 1 kg/animal/day and had unlimited access to tap water (23.5 mg S/L). Animals were observed daily for any overt clinical signs. Animals showing abnormalities were referred to the clinician for evaluation. The trial was terminated after 14 weeks when all remaining sheep were euthanized using intravenous barbiturate. Post-mortem and histopathological examination of all animals was undertaken.

**Sample collection.** Blood samples were obtained from the jugular vein during weeks 1, 5, 8, 10, 11, 12, and 13. Tissues (liver and frontal part of the brain) were obtained at necropsy. All specimens were stored at -70°C until analysis.

**Analytical procedure.** For measurement of TK activity and thiamine concentration, 1 g samples of tissues (liver and brain) were homogenized in 3 mL glycylglycine buffer (pH 7.6). Blood was hemolyzed by freezing at -70°C. Tissue homogenates and blood hemolysates were centrifuged at 2000g for 15 min and

the supernatants used for the assay. The TK assays were carried out as described by Massod et al (1971). Tissues TK activities were expressed as  $\mu\text{mol}$  sedoheptulose  $\text{min}^{-1} \text{g}^{-1}$  protein. Protein content in tissue supernatants was measured by the method of Lowry et al (1951). The red blood cells (RBC) TK activities are expressed as  $\mu\text{mol}$  sedoheptulose  $\text{min}^{-1} \text{g}^{-1}$  hemoglobin (Hb). Hemoglobin concentration in blood hemolysates was determined using a kit method (Sigma diagnostic procedure No. 525, St. Louis, MO). The percentage increase in TK activity after addition of thiamine pyrophosphate (TPP) to the assay is expressed as the TPP effect. Thiamine was measured as described by Olkowski and Gooneratne (1991).

**Histopathology.** Brains were fixed in 10% buffered formalin and after fixation were sliced transversely. Slices taken were routinely processed, embedded in paraffin, sectioned at  $0.5 \mu\text{m}$  and then stained with hematoxylin and eosin.

**Statistics.** Statistical analysis was carried out using the General Linear Model procedures of the Statistical Analysis System (1990). Repeated measures analysis of variance was used to identify main effects and interactions on blood variables. Polynomial contrasts were used to identify time trends (Snedecor and Cochran 1989). Differences within a group between time periods were analyzed using analysis of variance of the contrast variable. Analysis of variance (factorial) was used to analyze main effects and interactions on tissue variables. When the F-test was significant, Fisher's LSD test (Milliken and Johnson 1984) was used to determine differences among means. The morbidity and mortality significance

was evaluated by using the two-tail Fisher's exact test. Statistical significance was assumed to exist when the probability of making type I error was less than 0.05.

## RESULTS

**Clinical observations.** Of the 22 lambs fed the HS-LB<sub>1</sub> diet, seven developed clinical signs between the 3rd and 8th week of the trial. Initial signs consisted of loss of appetite, transient attacks of mild excitation and restlessness. Affected animals were not treated with thiamine. Two lambs recovered completely from the initial clinical signs. In the remaining five lambs, the disease progressed to incoordination, head pressing, and attacks of violent convulsions. Two lambs died and three that were *in extremis* were euthanized. One of the euthanized lambs had been given a tentative clinical diagnosis of Listeriosis. In addition to signs listed above, this animal had a head tilt to the left and had facial paralysis. No organisms were cultured from the cerebrospinal fluid and PEM was the definitive diagnosis made at post mortem examination. None of the LS or HS-HB<sub>1</sub> lambs developed clinical signs of PEM. Morbidity ( $p < 0.013$ ) and mortality ( $p = 0.08$ ) was attributed to lack of thiamine supplement in the HS-LB<sub>1</sub> diet.

**Gross pathology and histopathology.** A gross examination of brain slices revealed advanced necrotic lesions in 12 out of 22 brains from animals fed the HS-LB<sub>1</sub> diet. Extensive multiple white-yellowish foci of necrosis, some in a laminar pattern, were seen in the cortex, midbrain, and brain stem (Fig.1). The necrotic areas fluoresced under the ultraviolet light. The lesions were seen in the seven sheep which developed clinical signs of PEM and five others showing no

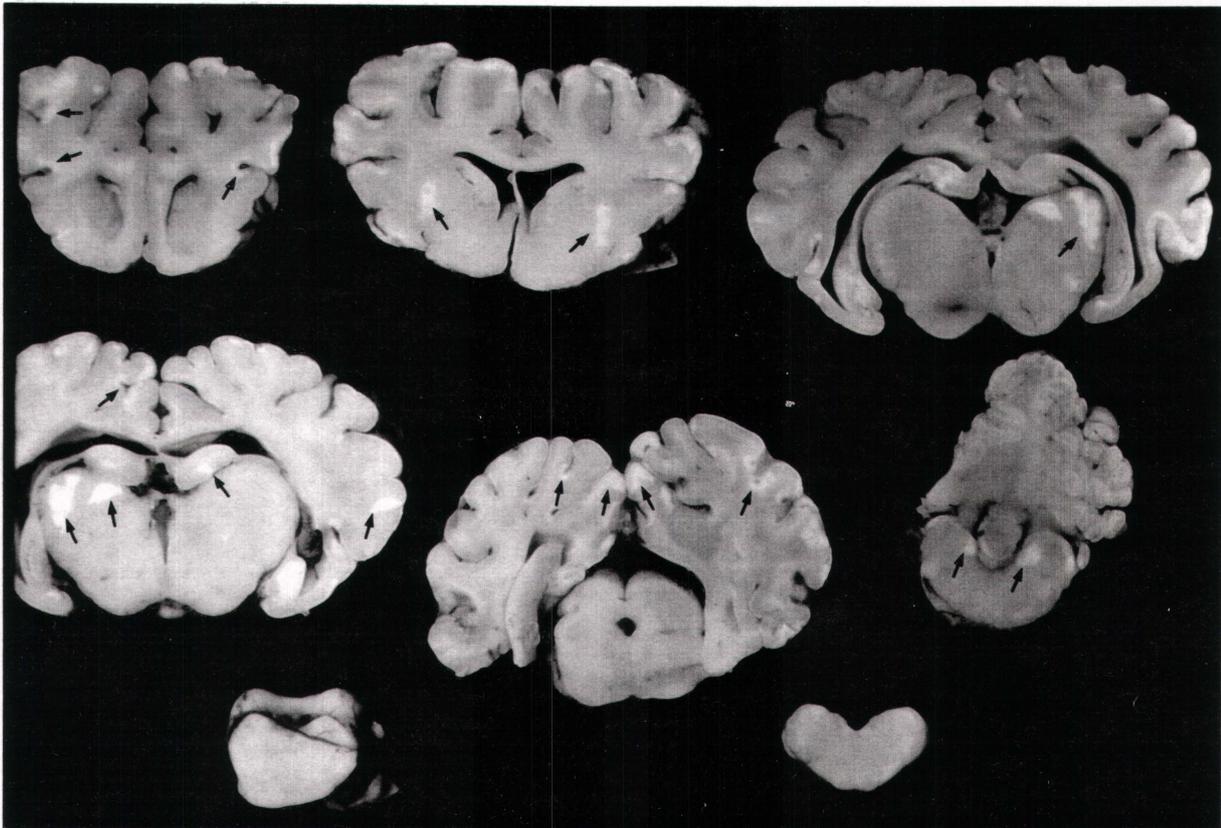


Fig. 1. Sections of brain from a lamb fed the HS-LB<sub>1</sub> diet. The lamb developed clinical signs of PEM during the fifth week of exposure and was euthanized. Note multiple fluorescing malacic areas (arrows) in various parts of the brain. Photographed using ultraviolet light.

apparent clinical signs. Such gross lesions were not seen in animals receiving either the HS-HB<sub>1</sub> diet or LS diets. Microscopic brain lesions were found in animals from both HS groups. The cerebral cortex appeared to be most affected. The necrotic lesions involved gyri and sulci, extending in some areas to the sides and depth of the sulci. For a detailed depiction of post mortem findings, see Rousseaux et al (1991).

**Thiamine status.** Tissue thiamine concentrations are shown in Table I. Liver thiamine differed among groups ( $p < 0.0001$ ) and was lowest in animals fed the LS-LB<sub>1</sub> diet. There were no differences ( $p > 0.05$ ) in liver thiamine concentration between LS-HB<sub>1</sub> and HS-LB<sub>1</sub> groups or between both thiamine supplemented groups. The HS-HB<sub>1</sub> group had a higher ( $p < 0.05$ ) thiamine concentration in liver than the HS-LB<sub>1</sub> group. Factorial analysis revealed the effects of dietary thiamine ( $p < 0.0001$ ) and sulphur ( $p < 0.002$ ) on liver thiamine concentration. There was no difference among the experimental groups for cerebral cortex thiamine concentration. There was strong effect of dietary thiamine ( $p < 0.0001$ ) on blood thiamine concentration. Blood thiamine concentration (Fig.2) did not differ among groups at week 1 of the experiment, but at weeks 5, 8, 10, 11, 12, and 13 was higher in animals fed the thiamine supplemented diets. There was a linear time trend for dietary factors; thiamine ( $p < 0.002$ ) and thiamine - sulphur interactions ( $p = 0.08$ ), and a quadratic time trend for dietary thiamine ( $p < 0.007$ ).

**Transketolase Activity and TPP effect.** No difference ( $p > 0.05$ ) was observed among treatment groups in TK activity of the liver or cerebral cortex (Table I).

Table I. Thiamine concentration, transketolase (TK) activity and TPP effect in liver and cerebral cortex from sheep fed diets containing normal (LS) or high (HS) levels of sulphur, unsupplemented (LB<sub>1</sub>) or supplemented (HB<sub>1</sub>) with thiamine.

Thiamine <sup>†</sup>	Liver		Cerebral Cortex	
	LB <sub>1</sub>	HB <sub>1</sub>	LB <sub>1</sub>	HB <sub>1</sub>
LS	29.80 (3.35)	45.16 (3.35)	23.01 (1.52)	25.63 (1.52)
HS	41.34 (2.14)	52.24 (2.51)	24.34 (0.97)	27.49 (1.14)
TK <sup>‡</sup>				
LS	4.06 (0.35)	4.17 (0.35)	11.50 (0.82)	11.57 (0.82)
HS	3.74 (0.23)	4.53 (0.26)	10.52 (0.53)	12.02 (0.62)
TPP <sup>§</sup>				
LS	14.47 (4.31)	21.52 (4.31)	12.81 (3.04)	10.90 (3.04)
HS	22.17 (2.82)	23.38 (3.24)	13.41 (1.95)	12.75 (2.28)

† g/kg tissue protein

‡ TK activity expressed as  $\mu\text{mol}$  of sedoheptulose  $\text{min}^{-1} \text{g}^{-1}$  protein

§ % increase in TK activity after addition of thiamine pyrophosphate

Values represent means (SE) of 9, 9, 22 and 16 sheep from LS-LB<sub>1</sub>, LS-HB<sub>1</sub>, HS-LB<sub>1</sub>, and HS-HB<sub>1</sub> group respectively.

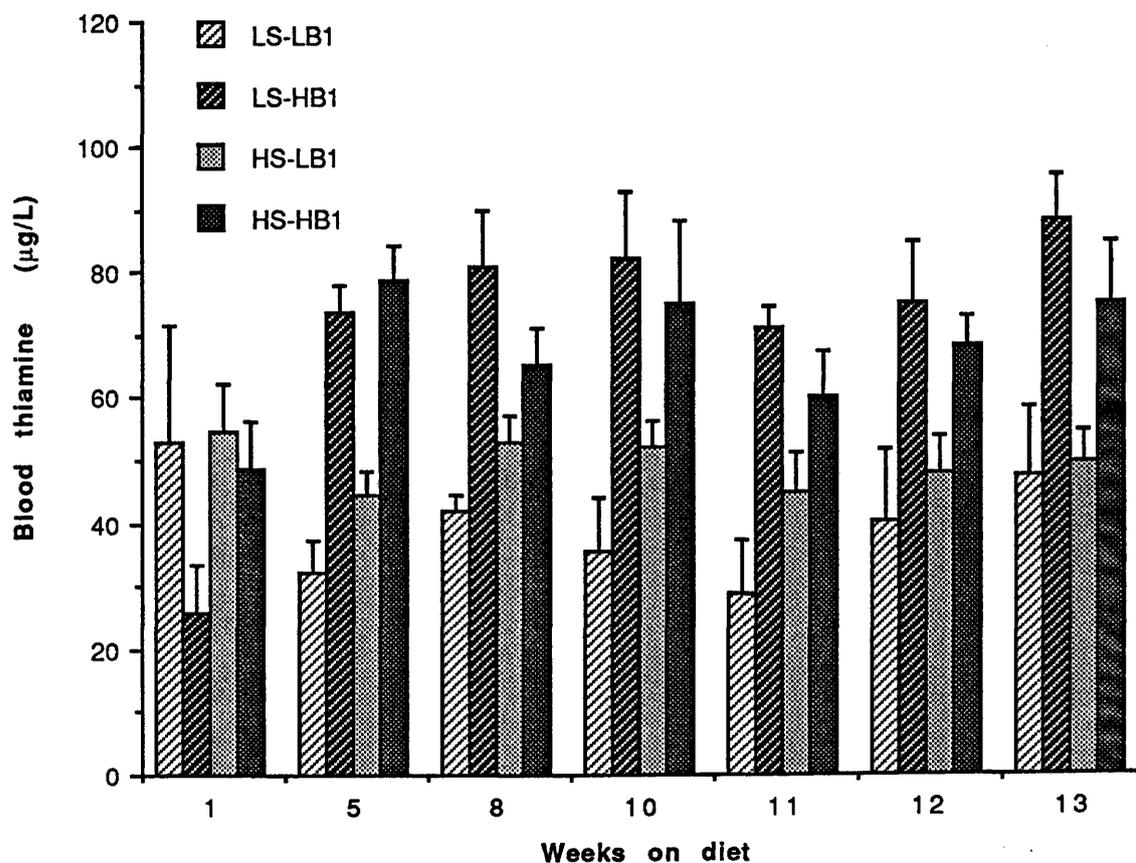


Fig. 2. Blood thiamine concentration in sheep fed diets containing normal level (LS) or excess (HS) S, supplemented (HB<sub>1</sub>) and unsupplemented (LB<sub>1</sub>) with thiamine. The bars represent means  $\pm$  SE of 5, 5, 9 and 15 sheep from LS-LB<sub>1</sub>, LS-HB<sub>1</sub>, HS-LB<sub>1</sub>, and HS-HB<sub>1</sub> group respectively.

Erythrocyte TK activity increased ( $P < 0.005$ ) in the LS-HB<sub>1</sub> group and ( $p < 0.003$ ) in the HS-HB<sub>1</sub> group (Fig.3). There were linear ( $p < 0.003$ ) and quadratic ( $p < 0.002$ ) time trends for dietary thiamine on erythrocytes TK activity. The erythrocyte TPP effect increased ( $p < 0.0001$ ) in the HS-LB<sub>1</sub> group and ( $p < 0.006$ ) in the HS-HB<sub>1</sub> group (Fig.4). Factorial analysis showed a strong ( $p < 0.0001$ ) effect of dietary thiamine in preventing an increase in TPP effect. There was no demonstrable influence of dietary S on TK activity or TPP effect.

## DISCUSSION

Historically, PEM (CCN) has been regarded as an effect of thiamine deficiency (Daly 1968, Edwin et al 1968, Edwin and Jackman 1973, Edwin et al 1979). However, it is important to stress that several diseases with similar clinical signs and histopathological features may have different causative factors (Edwin et al. 1979), and it is not known whether there is any common etiology.

Thornber et al (1979) experimentally induced PEM in pre-ruminant lambs by dietary thiamine deprivation. Clinical signs and brain lesions similar to PEM have been reproduced in various species with the use of the thiamine analogue, amprolium (Loew and Dunlop 1972, Lilja 1973, Markson et al 1974). In the present experiment, seven lambs fed a diet containing 0.63% S unsupplemented with thiamine developed PEM. Oral thiamine administration is known to be effective in the treatment of PEM (Thomas 1986). However, in our study, dietary thiamine supplementation prevented development of clinical signs but it did not totally prevent the development of brain lesions.

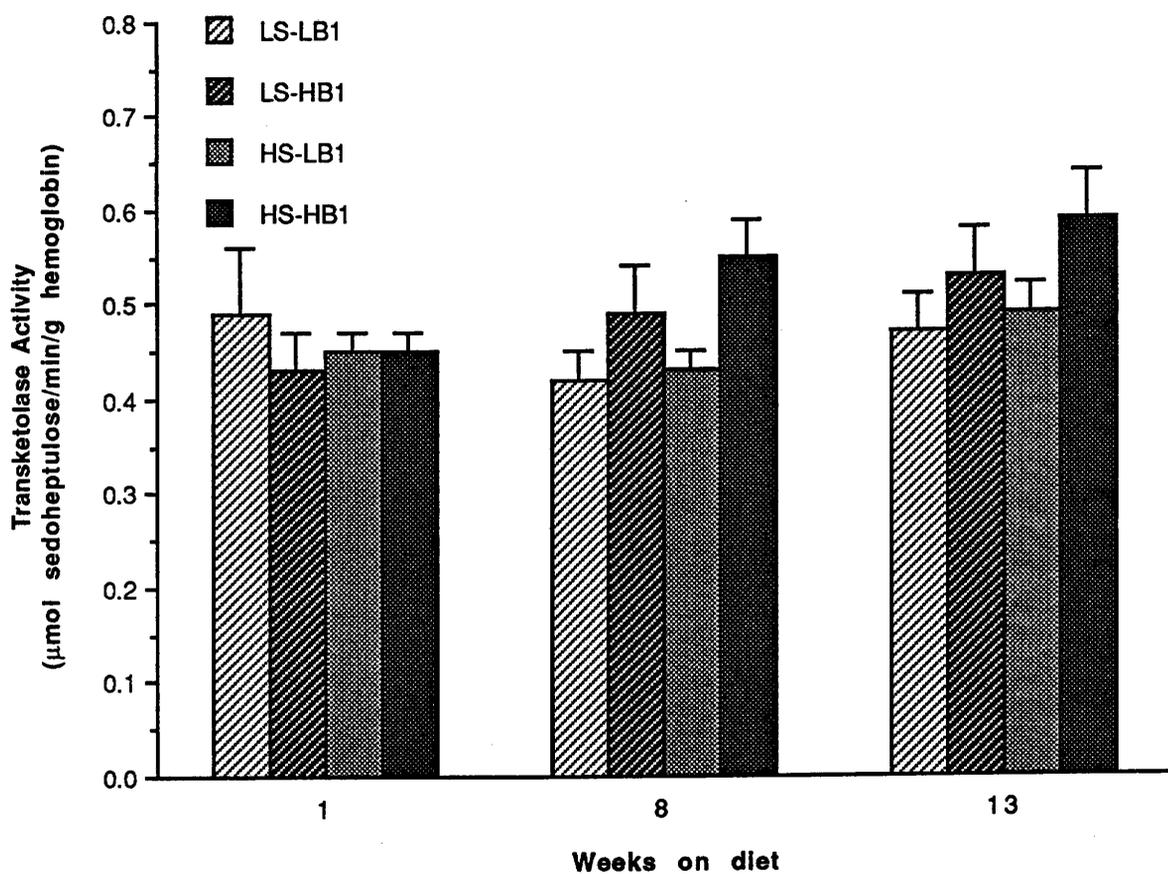


Fig. 3. Erythrocytes transketolase (TK) activity in sheep fed diets containing normal (LS) or excess (HS) S, supplemented (HB<sub>1</sub>) and unsupplemented (LB<sub>1</sub>) with thiamine. The bars represent means  $\pm$  SE of 5, 5, 9 and 15 sheep from LS-LB<sub>1</sub>, LS-HB<sub>1</sub>, HS-LB<sub>1</sub>, and HS-HB<sub>1</sub> group respectively.

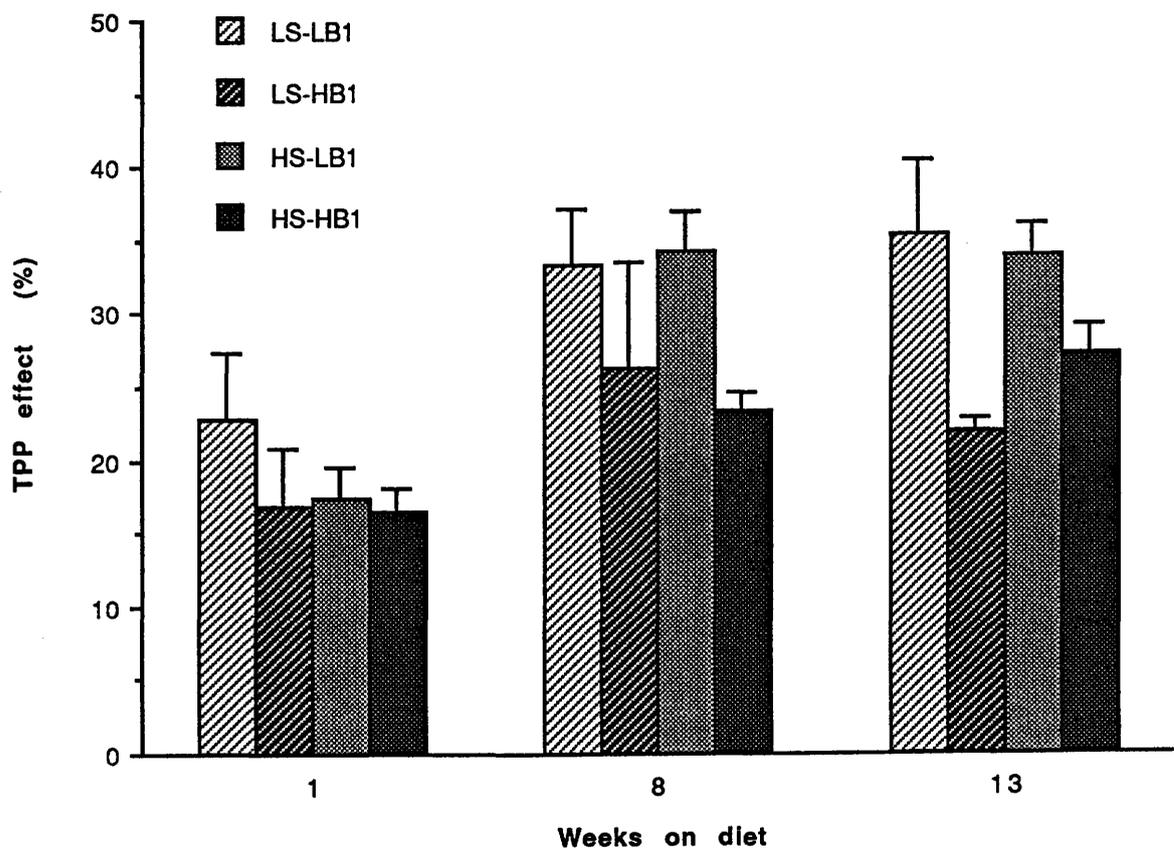


Fig. 4. Erythrocytes transketolase (TK) TPP effect in sheep fed diets containing normal (LS) or excess (HS) S, supplemented (HB<sub>1</sub>) and unsupplemented (LB<sub>1</sub>) with thiamine. The bars represent means  $\pm$  SE of 5, 5, 9 and 15 sheep from LS-LB<sub>1</sub>, LS-HB<sub>1</sub>, HS-LB<sub>1</sub>, and HS-HB<sub>1</sub> group respectively.

It appears that structural brain damage in PEM may not always manifest itself as a functional defect.

The occurrence of PEM lesions in the absence of clinical signs in farm animals is not well documented. Prior to this, only the study by Thornber et al (1979) reported mild localized brain lesions in thiamine deprived lambs killed before the development of clinical signs. It is notable that in our experiment, thiamine supplementation prevented development of clinical signs in animals with morphological changes in the brain. The distinctive feature present in clinically affected sheep was severe brain edema (Rousseaux et al 1991). The manifestation of acute clinical signs in the present study may be not an effect of necrotic lesions but rather an effect of severe brain edema which is a prominent feature in severe cases of PEM. It appears that higher concentrations of blood thiamine were required to prevent clinical manifestation of S toxicity. Hence, excess thiamine may be indispensable in preventing brain oedema, by increasing activity of the adenosine triphosphate dependent sodium pump (Matsuda and Iwata 1987), regulating ion uptake (Bettendorff et al 1990) and promoting expulsion of cellular water (Brown 1982).

The pathogenesis of S induced PEM is unknown. Excess dietary S can decrease blood thiamine concentration (Goetsch and Owens 1987, Gooneratne et al 1989a), and thiamine deficiency is sufficient to account for signs and lesions of PEM (Thornber et al 1979), albeit unlikely that this is the sole cause of S induced PEM in sheep. Indeed, in the present study, liver and blood thiamine concentrations

in sheep fed the HS-LB<sub>1</sub> diet were in fact slightly higher than in sheep fed the LS-LB<sub>1</sub> diet. This could indicate increased demand for thiamine in animals fed excess dietary S.

Sheep fed the HS diets could have a greater demand for thiamine and thus a higher TK activity. Results supporting this are; a) dietary factor S caused an increase in liver thiamine content, b) erythrocyte TK activity was highest in sheep receiving the HS-HB<sub>1</sub> diet. Furthermore, supplementation with thiamine increased erythrocyte TK activity and had a sparing effect on the TPP effect. Hence, if S toxicity does result in higher demand for thiamine, metabolic thiamine deficiency in the brain cannot be excluded on the basis of the present results. While there has been a general consensus that thiamine inadequacy is involved in the etiology of PEM, the mechanism by which this occurs is unknown. Increased metabolic demand is one of the possible considerations, and in this scenario affected animals (showing no difference in thiamine status in comparison to normal animals) can in fact be deficient.

The direct toxic effect of S metabolites can be an etiological factor in S induced PEM. In-vitro, sulphite derived radicals can be formed through a catalytic reaction of peroxidases and are postulated to cause lipid peroxidation and damage to the biological membrane (Mottley and Mason 1988). Since rumen sulphate is reduced to sulphite and sulphide (Lewis 1956), and absorbed sulphide is oxidised to sulphite and sulphate in the body and recycled to the rumen

(Kennedy et al 1975), there is a potential for sustained synthesis of S-containing free radicals both in the rumen and systemically.

The toxic effects of excess dietary S are unique in ruminants. The brain is the target organ. Brain tissue, because of its high lipid content would be extremely vulnerable to damage by free radicals. Furthermore, considering that the neural tissue has substantially less damage repairing and regenerating ability than other tissues, it is possible that even a mild thiamine insufficiency, under conditions of increased requirement, may lead to impairment of detoxifying processes and can result in brain damage. It has been shown that a deficit of thiamine increases the intensity of lipid peroxidation (Lychko et al 1987). Free thiamine is thought to protect the cell by scavenging potentially toxic intermediates generated by the myeloperoxidase/H<sub>2</sub>O<sub>2</sub>/halide system (Theron et al 1981).

In summary, this study indicates that S induced PEM is not likely caused by overt thiamine deficiency based on commonly used criteria, but it appears that thiamine plays a major role in prevention of clinical signs and alleviation of PEM lesions. Perhaps thiamine deficiency in the brain may not be reflected exclusively by lowered enzyme activity. A study by Gibson et al (1989) indicates that in pyriethamine induced thiamine deficiency in rats, selective cellular responses cannot be related to enzymatic alterations. Thiamine may play some protective role apart from its metabolic effect through thiamine dependent enzymes and, in the situation of increased demand for thiamine, even a mild thiamine deficiency can lead to impairment in detoxifying processes and resultant brain damage.

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**THE EFFECTS OF COPPER STATUS ON THIAMINE METABOLISM  
IN SHEEP FED A HIGH SULFUR DIET**

A.A. Olkowski, S. R. Gooneratne, and D.A. Christensen. *Can. J. Anim. Sci.* 1991; 71:813-824.

**ABSTRACT**

The effect of Cu status on selected aspects of thiamine metabolism was studied in sheep exposed to high dietary S over a period of fourteen weeks. Nine cross-bred 2 month old female lambs were used. The animals were divided into three groups (n=3) and offered a basal diet containing 0.63% S (0.45% as sulfate) and 14 mg thiamine kg<sup>-1</sup> DM. Copper and Mo were variable ingredients. Sheep in group 1 received a 14 mg Cu kg<sup>-1</sup> DM diet, whereas those in groups 2 and 3 received a 6 mg Cu kg<sup>-1</sup> DM diet. To precipitate Cu insufficiency, group 3 received an additional 20 mg Mo kg<sup>-1</sup> DM diet. The concentration of blood thiamine tended to increase in sheep from group 1, but decline in sheep from group 3 which also had a lower concentration of Cu in plasma. In these groups, a similar trend was observed in thiamine and Cu concentrations of duodenal fluid. Liver thiamine tended to be higher (P=0.09) in group 3 sheep than in groups 1 and 2. However, the concentration of thiamine in the kidney and cerebral cortex did not differ among the groups. Copper concentration was lowest in the liver and brain, but highest in the kidney of sheep from group 3. The erythrocyte thiamine pyrophosphate (TPP) effect increased (P<0.035) in sheep

in groups 2 and 3 during the 8th week on the diet and in all sheep during the 13th week. We conclude that Cu plays an important role in the metabolism of thiamine in the gastrointestinal tract, as well as systemically.

## INTRODUCTION

Excessive S intake, as a consequence of high S concentration in both feed and drinking water, prevails in many parts of North America (Raisbeck 1982; Boila 1988; Harries 1987; Gooneratne et al. 1989a). Detrimental effects of high dietary intake of S on the metabolism of both Cu (Suttle 1974, Smart et al. 1986) and thiamine (Goetsch and Owens 1987; Gooneratne et al. 1989a) in ruminants have been observed. An increase in dietary S (organic and inorganic forms) in ruminants limits the availability of Cu through formation of insoluble Cu sulfide in the alimentary tract (Suttle 1974). When a high concentration of S and Mo are present in the diet of ruminants the availability of Cu to the animal is considerably decreased (Allen and Gawthorne 1987).

A causal relationship has been established between excessive intake of S and polioencephalomalacia (PEM) (Raisbeck 1982; Sadler et al. 1983; Gooneratne et al. 1989a). Polioencephalomalacia is a central nervous system (CNS) disorder commonly attributed to thiamine deficiency (Jensen et al. 1956; Daly 1968). The recommended dietary S level for ruminants is 0.16 - 0.24% (National Research Council 1984, 1985). At a level of 0.5%, dietary S can induce PEM in cattle (Gooneratne et al. 1989a).

Concurrent thiamine and Cu deficiencies can occur in feedlot cattle that consume water with a high sulfate content (Gooneratne et al. 1989a). The latter authors observed that both Cu and thiamine deficiency occurring under these circumstances can be corrected by dietary supplementation of Cu alone. At present, the mechanisms of S-Cu-thiamine interactions in ruminants are not well understood.

The present experiment was an exploratory study undertaken to examine the effects of Cu status on thiamine metabolism in ruminants receiving excess S in the diet. Molybdenum was used to induce Cu deficiency. The main objective of this study was to identify possible interactions between these nutrients and provide a background for future study.

## **MATERIALS AND METHODS**

**Animals and Diets.** Nine cross-bred female lambs approximately 8 weeks old,  $14.6 \pm 0.5$  kg BW (mean  $\pm$  SE), were randomly divided into three groups (n=3). During the first 6 weeks of the trial the lambs were grouped in pens. Thereafter, the lambs were housed in individual crates. The lambs were each fed 1 kg of diet given as a single meal at 8:00 and had free access to tap water throughout the experiment. This level of feeding was chosen to maintain a constant and uniform intake of elements under test. Feed intake was monitored daily. The basal diet, comprised of barley (59%) - soybean meal (5%) - alfalfa (32%) - tallow (1%) - minerals and vitamins (3%), was prepared to meet nutritional requirements recommended by the National Research Council (1985). The basal diet contained

6 mg Cu and 5 mg Mo kg<sup>-1</sup> DM and 0.63% S, where 0.45% S was from K,Mg sulfate (Dynamate, IMC. Mundelein, IL). The experimental diets were: basal, Cu supplemented, and Mo supplemented. Copper and Mo were added to the basal diet as Cu oxide and ammonium molybdate respectively. The diets were pelleted (4-5 mm). Group 1 was fed a diet containing 14 mg kg<sup>-1</sup> DM of Cu (Cu supplemented). Group 2 received the basal diet. Lambs from group 3 received a diet containing 25 mg Mo kg<sup>-1</sup> DM (Mo supplemented). The thiamine concentration in all diets was 14 mg kg<sup>-1</sup> DM. After 8 weeks of adaptation to the diet, the gall bladder was cannulated and a re-entrant cannula was placed in the mid-third of the duodenum in all sheep. The surgery was carried out according to the procedure described by Caple and Heath (1972). The trial was terminated during the 14th week when all sheep were euthanized with intravenous barbiturate. Care of animals was administered in accordance with the guidelines presented by the Canadian Council on Animal Care (1980).

**Sample Collection.** The collection of specimen samples from each animal started between 9:30 and 10:00 on the same day of each sampling week. Blood samples were obtained from the jugular vein prior to the start of the trial (week 0) and, thereafter, on weeks 5, 8, 10, 11, 12 and 13. Bile and urine were sampled for 6 h, once a week, during weeks; 10, 11, 12 and 13 of the trial. On the day of sampling, the bladder was catheterized with an inflatable Foley catheter (12 Fr/30 cc, Inmed Corporation, Nercross, GA). Urine was collected into plastic bags for 6 h, volume was recorded and a 6 mL sample was taken. In order to maintain

entero-hepatic bile circulation, the 6 h bile sampling was divided into 12 collection periods. During each period bile was collected for 30 min into plastic bags. At each collection the volume was recorded and a 3-mL sample was taken while the remaining bile was returned into the duodenum. Two 30 min bile samples were pooled in proportions corresponding to respective total volumes to form a 1 h sample. Duodenal digesta (10 mL) was collected on week 13. The digesta was centrifuged at 2000 g for 10 min and the fluid was used for analysis. Tissues (liver, kidney, and brain) were collected at necropsy. All specimens were stored at -70°C until analysis.

### **Analytical Procedures**

**Mineral analysis.** Plasma Cu and Zn concentrations were measured in the trichloroacetic acid (TCA) soluble fraction as outlined by Smith and Wright (1975). Samples of duodenal fluid were mixed with 20% TCA and deionized water at a ratio of 1:1:3 v/v and were centrifuged at 2000 g for 15 min. Copper and Zn concentrations were measured in the supernatants. This procedure was used to ensure measurement of bioavailable Cu in the duodenal fluid. Copper and Zn concentration in the bile and urine was measured directly after appropriate dilution with deionized water. Tissues for Cu and Zn analysis were freeze dried. Feed and tissues were digested in a mixture of nitric/perchloric acids (7:3 v/v). Copper, Zn and Mo were measured on an atomic absorption spectrophotometer (Perkin Elmer model 5000). Sulfur in feed was analyzed by induction coupled plasma emission spectroscopy (ARL model 3410).

**Transketolase (TK) analysis.** For measurement of TK activity, 1 g samples of tissues (liver, kidney, and brain) were homogenized in 3 mL glycylglycine buffer (pH 7.6). Blood was hemolyzed by freezing at  $-70^{\circ}\text{C}$ . Tissue homogenates and blood hemolysates were centrifuged at 2000 g for 15 min and the supernatants used for the assay. The TK assays were carried out as described by Massod et al. (1971). Tissues TK activities were expressed as  $\mu\text{mol sedoheptulose min}^{-1} \text{g}^{-1}$  protein. Protein content in tissue supernatants was measured by the method of Lowry et al. (1951). The TK activities in red blood cells (RBC) are expressed as  $\mu\text{mol sedoheptulose min}^{-1} \text{g}^{-1}$  hemoglobin (Hb). Hemoglobin concentration in blood hemolysates was determined using a kit method (Sigma diagnostic procedure No. 525). The percentage increase in TK activity after addition of thiamine pyrophosphate (TPP) to the assay is expressed as the TPP effect.

**Thiamine analysis.** Thiamine was measured as described by Olkowski and Gooneratne (1991).

**Statistics.** Statistical analysis was carried out using the General Linear Model procedures of SAS (SAS Institute 1990). Missing data points were replaced with fitted values calculated using regression. Repeated measures analysis of variance was used to identify main effects and interactions. Polynomial contrasts were used to identify time trends (Snedecor and Cochran 1989). Time-treatment interactions and time trends were further examined using contrasts. The contrasts of interest were: groups 1 vs 2, 1 vs 3, 2 vs 3. Differences within a group between time periods were analyzed using analysis of variance of the contrast variable.

One way analysis of variance was used to compare groups within the time period. Tukey's HSD test (Miller 1980) was used to determine differences between means. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

## RESULTS

**Feed Intake.** Feed intake did not vary with treatment. There was a temporary reduction in feed intake during the first week after surgery. Otherwise the feed intake was consistent throughout the experiment

**Thiamine Status.** Blood thiamine concentrations in sheep in groups 1, 2, and 3 are given in Figures 1a, 1b, and 1c respectively. There was considerable variation in blood thiamine at the start of the experiment (mean  $59.6 \pm \text{SE } 10.2 \mu\text{g L}^{-1}$ ) with a coefficient of variation 49%. The time x treatment interaction was found ( $P < 0.016$ ) with the contrasts between groups being: 1 vs 2 ( $p = 0.06$ ), 1 vs 3 ( $P < 0.04$ ), 2 vs 3 ( $P < 0.02$ ). There was a tendency ( $P = 0.087$ ) towards a linear time-trend. Blood thiamine concentration tended to increase in group 1 sheep and decline in group 3. Groups 1 and 3 had different ( $P < 0.035$ ) slopes. The group 2 blood thiamine profile was distinctly different from groups 1 and 3. The concentration of blood thiamine in this group declined at week 5, then increased. A third order polynomial showed that group 2 differed ( $P < 0.02$ ) from groups 1 and 3. A fourth order polynomial was required to adequately fit both the mean across groups ( $P < 0.003$ ) and the individual groups ( $P < 0.02$ ). The 2nd, 5th and 6th orders were not significant.

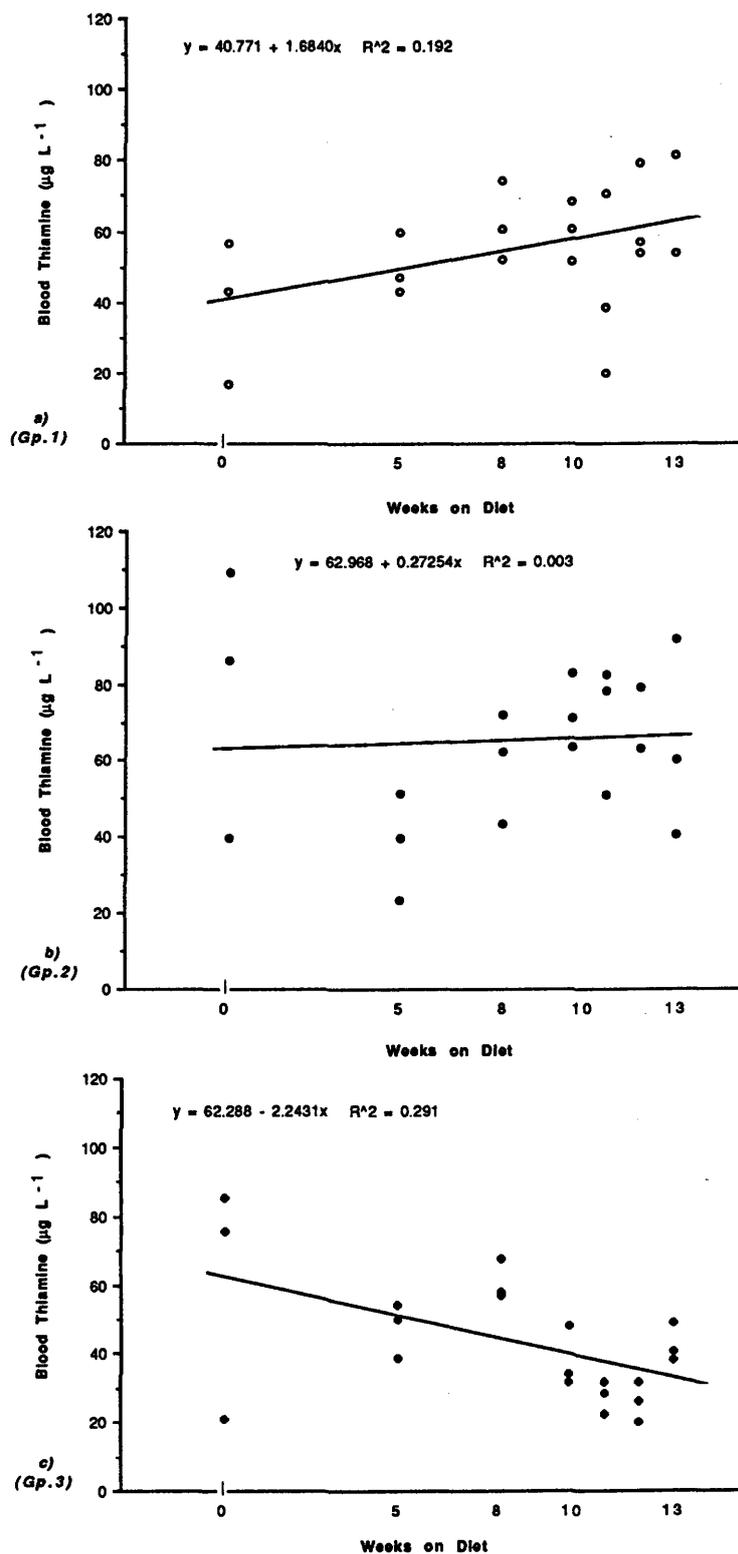


Fig. 1 a,b,c. Scatter plots of blood thiamine concentrations in sheep fed high S diet with variable concentration of Cu and Mo. The regression lines indicate a time-trend of the treatment effect. Concentrations of variable ingredients in the diets ( $\text{kg}^{-1}$  DM) were: group 1 = 14 mg Cu; group 2 = 6 mg Cu; group 3 = 6 mg Cu, 25 mg Mo.

Thiamine concentration in duodenal fluid showed a trend, with the concentration in group 1 > group 2 > group 3 (Fig.2). The measured concentration was relative to the total amount. The liver thiamine concentration tended ( $P=0.09$ ) to be higher in group 3 (Table 1). Thiamine concentrations in the kidney and brain were similar among the groups. Urinary thiamine excretion did not differ among groups. The excretion (mean  $\pm$  SE) was  $126.7 \pm 12.5 \mu\text{g h}^{-1}$  with a coefficient of variation of 58%. There was a significant ( $P<0.01$ ) time  $\times$  treatment interaction for biliary thiamine excretion. The contrasts between groups differed: group 1 vs 2, and 2 vs 3 ( $P<0.03$  for both) and group 1 vs 3 ( $P<0.02$ ). The biliary excretion of thiamine (mean  $\pm$  SE  $\mu\text{g h}^{-1}$ ) were  $2.0 \pm 0.3$ ,  $1.48 \pm 0.1$  and  $1.23 \pm 0.21$  for groups 1, 2 and 3, respectively. Samples from week 13 were not available for biliary thiamine analysis.

**Transketolase Activity and TPP Effect.** Transketolase activities in the liver, kidney, and cerebral cortex were similar among the three groups of sheep. However, the TPP effect in the liver was higher in group 1 > group 3 > group 2. No changes were observed in the TPP effect in the kidney and cerebral cortex among the three groups of sheep.

There were no differences ( $P>0.05$ ) in RBC TK activity among the three groups. The RBC TK activity (mean  $\pm$  SE) was  $0.45 \pm 0.02 \mu\text{mol sedoheptulose min}^{-1} \text{g}^{-1} \text{Hb}$ . There was a time effect ( $P<0.001$ ) and a treatment  $\times$  time interaction ( $P<0.001$ ) for the TPP effect in RBC. The contrasts between groups differed: group 1 vs 2 ( $P<0.001$ ), 2 vs 3 ( $P<0.04$ ) and group 1 vs 3 ( $P<0.003$ ).

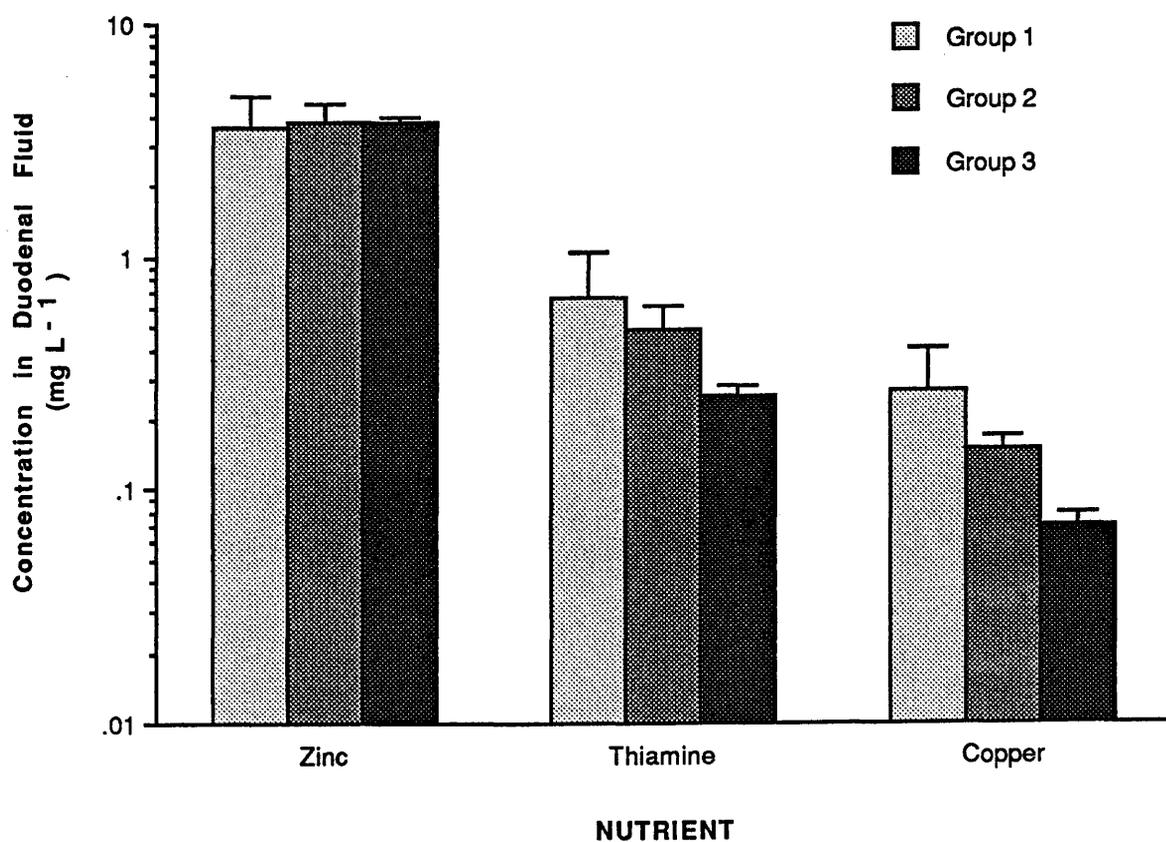


Fig. 2. Concentrations of thiamine, Cu and Zn in duodenal fluid collected at wk 13 from sheep fed high S diet with variable levels of Cu and Mo. Concentrations of variable ingredients in the diets (kg<sup>-1</sup> DM) were: group 1 = 14 mg Cu; group 2 = 6 mg Cu; group 3 = 6 mg Cu, 25 mg Mo.

Table 1. Concentration of Cu<sup>†</sup> and thiamine<sup>‡</sup> in liver, kidney cortex, and cerebral cortex of sheep fed excess of S in the diet with varied concentration<sup>§</sup> of Cu and Mo

Group	Tissue						
	Liver		Kidney cortex		Cerebral cortex		
	Thiamine	Cu	Thiamine	Cu	Thiamine	Cu	
1	37.1	283.4b	39.2	18.4a	21.8	10.2b	
2	36.7	139.9a	38.6	20.5a	22.6	10.8b	
3	54.2	34.9a	34.1	70.9b	23.2	8.6a	
SEM	5.2	38.9	4.0	11.7	1.7	0.4	
ANOVA	P <	0.09	0.01	NS	0.03	NS	0.02

† mg kg<sup>-1</sup> DM tissue

‡ g kg<sup>-1</sup> tissue protein

§ Concentration of variable ingredients in the diets (kg<sup>-1</sup> DM): group 1 = 14 mg Cu; group 2 = 6 mg Cu; group 3 = 6 mg Cu, 25 mg Mo.

SEM Standard error of the mean.

a,b Means with different letter within a column differ (P<0.05).

The TPP effect increased ( $P < 0.025$ ) during weeks 8 and 13 in sheep in groups 2 and 3, compared to the value at the start of the experiment (Fig. 3). The TPP effect in RBC in group 1 sheep during week 8 was similar to that at the start of the experiment (week 0), but increased ( $P < 0.035$ ) at week 13.

**Zinc Status.** Zinc concentrations were within the normal range in liver, kidney, brain and plasma. There were no differences among groups in Zn concentration in the tissue, plasma or duodenal fluid. Also biliary and urinary excretions were not different among groups.

**Copper Status.** There was a significant treatment effect ( $P < 0.018$ ) on plasma Cu concentration. The contrasts between groups were: 1 vs 3 ( $P < 0.043$ ), 2 vs 3 ( $P < 0.008$ ), 1 vs 2 ( $P > 0.05$ ). Plasma Cu concentration declined in sheep from all treatments at week 5 of the experiment, but only in animals from group 3 did it continue to be lower (Fig. 4). The treatment effect on biliary Cu excretion was also highly significant ( $P < 0.001$ ). Biliary Cu excretion was consistently highest in group 1 and lowest in group 3 animals during weeks 11, 12, and 13 (Table 2). Urinary Cu excretion in sheep in all three groups did not differ. The mean excretion was  $4.86 \mu\text{g h}^{-1} \pm 0.96$  (SE) with a coefficient of variation of 60%. Tissue Cu concentrations are shown in Table 1. Liver Cu concentration differed ( $P < 0.012$ ) among groups. Kidney and cerebral cortex Cu concentration of groups 1 and 2 sheep were within the normal range. The kidney Cu concentration of group 3 sheep was elevated ( $P < 0.032$ ), but the concentration of Cu in the cerebral cortex was lower ( $P < 0.017$ ) than in groups 1 and 2.

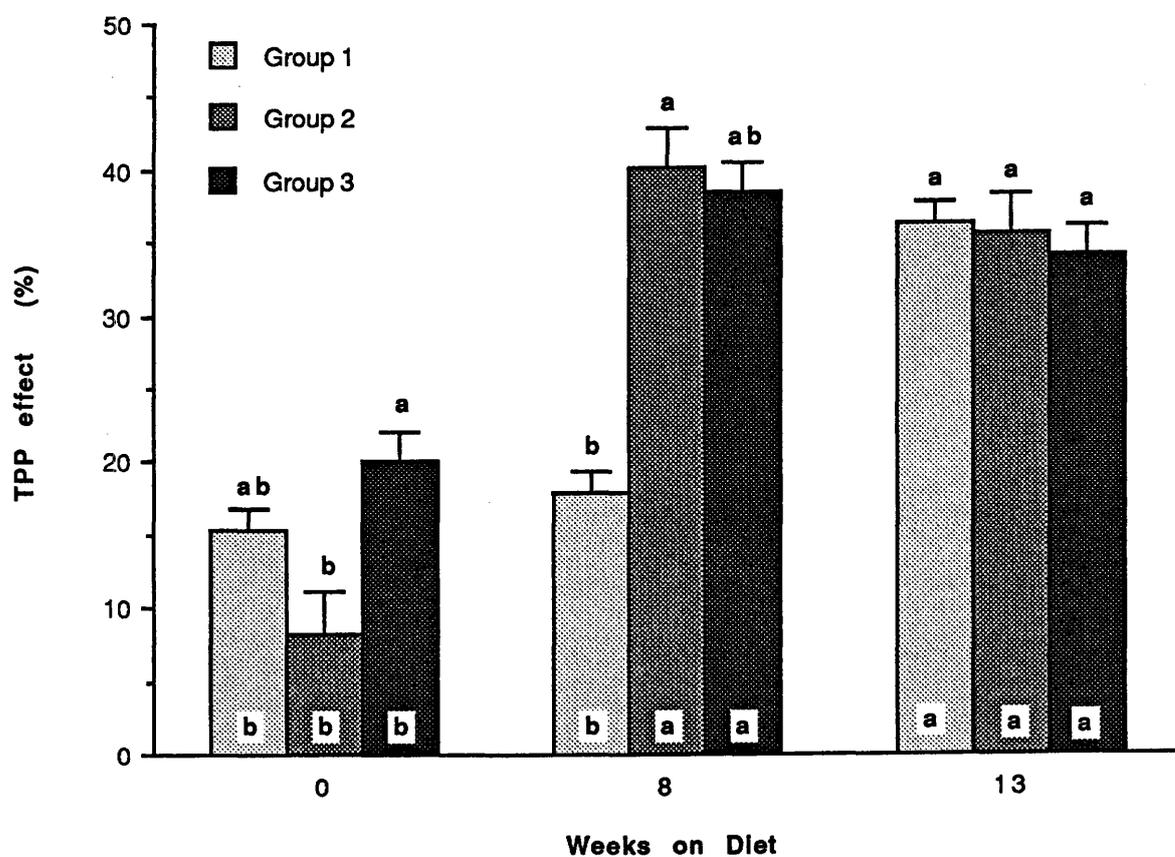


Fig. 3. TPP effect for RBC TK in sheep fed high S diets with variable concentrations of Cu and Mo at wk 0 and at wks 8 and 13. The letters on the bars indicate differences within groups. The TPP effect was higher ( $P < 0.03$ ) in groups 2 and 3 at wk 8 and in all groups at wk 13 ( $P < 0.035$ ), compared to the value at wk 0. The letters above the bars indicate differences ( $P < 0.05$ ) among groups. Concentrations of variable ingredients in the diets ( $\text{kg}^{-1}$  DM) were: group 1 = 14 mg Cu; group 2 = 6 mg Cu; group 3 = 6 mg Cu, 25 mg Mo.

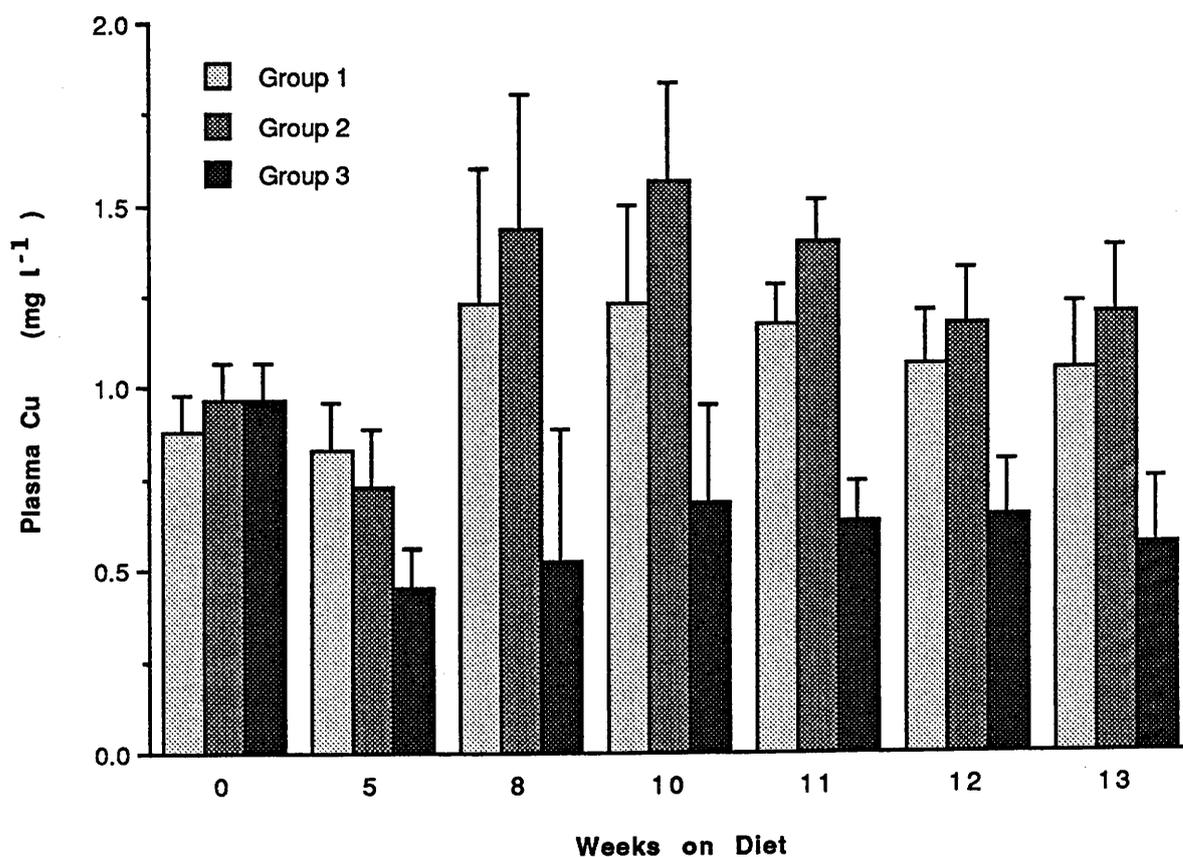


Fig. 4. Plasma Cu concentrations in sheep fed high S diets with variable concentrations of Cu and Mo. Concentrations of variable ingredients in the diets (kg<sup>-1</sup> DM) were: group 1 = 14 mg Cu; group 2 = 6 mg Cu; group 3 = 6 mg Cu, 25 mg Mo.

Table 2. Biliary Cu excretion<sup>†</sup> in sheep fed excess S in the diet with varied concentration<sup>‡</sup> of Cu and Mo

Group	Weeks on diet							
	10	(n)	11	(n)	12	(n)	13	(n)
1	7.2	(3)	18.5b	(2)	9.3b	(2)	11.6c	(2)
	±		±		±		±	
	2.5		2.0		1.1		0.7	
2	7.8	(3)	4.6a	(3)	5.6a	(3)	7.2b	(3)
	±		±		±		±	
	2.5		1.6		0.9		0.6	
3	3.9	(3)	3.1a	(3)	2.1a	(2)	1.5a	(3)
	±		±		±		±	
	2.5		1.6		1.1		0.5	
ANOVA	P<	NS	0.004		0.03		0.0002	

† Biliary Cu excretion is expressed as mean ± SEM, µg h<sup>-1</sup>

‡ Concentration of variable ingredients in the diets (kg<sup>-1</sup> DM): group 1 = 14 mg Cu; group 2 = 6 mg Cu; group 3 = 6 mg Cu, 25 mg Mo.

n number of animals sampled

a,b,c Means with different letter within a column differ (P<0.05).

Copper concentration in duodenal fluid showed a similar trend to liver Cu, with concentration in group 3 < group 2 < group 1 (Fig. 2). There was a correlation between duodenal Cu and thiamine concentration ( $r=0.96$ ), but not between Zn and thiamine ( $r=0.09$ ).

## DISCUSSION

In ruminants, excess dietary S can influence the metabolism of Cu (Suttle 1974; Christensen et al. 1984; Smart et al. 1986) and thiamine (Gooneratne et al. 1989a; Goetsch and Owens 1987). The results reported here demonstrate that a complex interaction exist among these nutrients.

The formation of Cu sulfides (CuS) in the gastrointestinal (GI) tract is thought to be the main cause of decreased Cu bioavailability in animals fed excess S (Suttle 1974). Excess dietary Mo in the presence of high S results in the formation of thiomolybdates (TM) that reduce the availability of Cu not only in the GI tract but also systemically (Price et al. 1987; Allen and Gawthorne 1987; Gooneratne et al. 1989c). In the present study, the concentration of TCA soluble Cu in plasma declined in sheep from all experimental groups at week 5 of the experiment. The concentration of Cu in plasma remained lower in group 3 animals throughout the duration of the study. A persistent decrease in the concentration of TCA soluble Cu in the plasma was observed in sheep treated with TM (Kincaid and White 1988). Lower TCA soluble Cu concentration in the duodenal fluid of group 3 versus group 2 sheep may have been caused by the formation of insoluble TM-Cu complexes (Allen and Gawthorne 1987) which would reside in the particulate

fraction of the digesta. Thiomolybdates formed in the rumen and passed to the duodenum are associated predominantly with the solid fraction of the digesta (Price et al. 1987). The effect of S and Mo appears to be specific for Cu, since no effect on Zn status was observed in the present study. Decreased solubility of Cu, but not Zn and Fe, is observed in the ruminal contents after the addition of Mo (Allen and Gawthorne 1987).

Lower concentrations of Cu in the brain and liver in group 3 could have resulted from decreased bioavailability of Cu caused by the formation of TM, which may bind Cu and prevent its absorption (Allen and Gawthorne 1987). Paradoxically, the kidneys of these sheep had the highest Cu residues while plasma Cu concentrations were lower than those from sheep in groups 1 and 2. This indicates a systemic effect of the dietary Mo and S. Presumably, some TM formed in the GI tract may have been absorbed since the effect observed here was in accord with previous experiments which reported reduced concentration of Cu in the liver and plasma and deposition of Cu in the kidney following oral (Kincaid and White 1988) and intravenous (Gooneratne et al. 1989b) TM administration.

Goetsch and Owens (1987) observed a reduced duodenal flow of thiamine and decreased thiamine concentration in the blood of cattle fed excess dietary sulfate. Copper was not measured in the latter study. In the present study, an increased dietary Cu concentration resulted in a higher thiamine concentration in duodenal fluid as well as in blood. In ruminants, dietary sulfate is reduced to sulfite and

sulfide by rumen microorganisms (Lewis 1956). Excess dietary Cu, by complexing the sulfide ions into insoluble CuS and/or TM-Cu, could enable the reduction of sulfite to move forward. This would hinder accumulation of sulfite ions in the rumen, thus preventing degradation of thiamine.

Sulfite ions degrade thiamine molecules by nucleophilic displacement (Leichter and Joslyn 1969). Raisbeck (1982) hypothesized that sulfite ions produced in the rumen are capable of destroying ruminal thiamine. Thiamine degradation in the rumen by nonenzymatic (thermostable) activity has been previously reported (Quaghebeur et al. 1975). Neither Cu nor S was measured in the latter study. The present study did not provide direct evidence of ruminal thiamine degradation. However, lower thiamine concentrations in the duodenal fluid in groups 2 and 3 would indicate such a decline in ruminal thiamine, with Cu apparently playing a preventative role. The thiamine concentration in the duodenal fluid was correlated with its Cu concentration ( $r=0.96$ ). This relationship appears to be specific since there was no correlation between Zn and thiamine concentrations in the duodenal fluid.

The thiamine molecule can undergo remarkable conformational transformations. Interconversion of the thiamine molecule to thiazole closed ring, tricyclic, or thiol forms has been described (Metzler and Maier 1962). Copper promotes formation of thiamine disulfide in-vitro (Kobayashi 1972). Thus, Cu by facilitating conformational change to a thiazole open ring, would protect the thiamine molecule against nucleophilic attack. Also, such a form could be further

stabilized by coupling to other sulfhydryl compounds (Metzler and Maier 1962). Indeed, certain disulfide derivatives of thiamine appear to be more bioavailable than thiamine itself (Duclos and Haake 1974; Davis and Icke 1983).

The present study indicates that Cu may play an important role in thiamine metabolism, not only in the GI tract but also systemically. Notably, higher concentration of thiamine in liver in group 3 sheep occurred in spite of low duodenal and blood thiamine concentration. Group 3 sheep also had the lowest liver Cu concentration. Hence, when the diet contains excessive amounts of both Mo and S, the Cu content of liver decreases and liver thiamine is stored in some unknown "nonavailable" form which cannot be utilized by the body to maintain blood thiamine, or be excreted in the bile. This suggests that Cu status may be important in the release of liver thiamine. However, the role of Mo is unclear.

Hill et al. (1988) proposed a reference range of 25 to 63  $\mu\text{g L}^{-1}$  for blood thiamine concentration in normal sheep and cattle, and a level below 18  $\mu\text{g L}^{-1}$  as indicative of deficiency. In the present study, blood thiamine concentration remained above this level. However, these values were established for animals of normal mineral status grazing pasture. The requirement for thiamine may be higher in animals fed excess S. In the present study, higher values of the TPP effect indicated thiamine deficiency in sheep from groups 2 and 3 at week 8 of the trial but not in sheep from group 1. However, all three groups were affected similarly at week 13. The RBC TK activity and TPP effect are useful indices of thiamine status (Bogin et al. 1985; Ali et al. 1987). Hence, the increase in RBC

TPP effect may be a reflection of a higher demand for TK and consequently a higher requirement for thiamine, probably due to the unknown "toxic" effects of S.

The systemic effect of excess dietary S is not well understood. Weeth and Hunter (1971), who investigated extensively the effects of sulfate-water on several physiological parameters and blood chemistry, concluded that a specific toxic effect of sulfate was not apparent, however, the adverse effects were related to the sulfate ion. Also, not all the detrimental effects of high dietary S can be explained by Cu deficiency. According to Smart et al. (1986), the high mortality rate among calves attributed to high sulfate in drinking water may have been related to factors other than Cu status.

Cramer et al. (1984) suggested that thiamine may play a role in the bioregulation of Cu. A biological interrelationship between dietary thiamine and Cu was shown in rats (Ellerson and Hilker 1985). The latter authors showed that the concentration of Cu in liver was decreased by high dietary thiamine in the low and high Cu groups, but was increased in the normal Cu group. In cattle with low blood concentrations of both Cu and thiamine, improvement in Cu status from deficient to adequate resulted in a marked increase in blood thiamine concentration (Gooneratne et al 1989a). Apparently, these two nutrients may interact in a reciprocal fashion. However, the molecular basis of this interaction is not known.

Copper may play a role in the coordination of the coenzyme to fit the stereochemistry of the catalytic center of the apoenzyme. There is evidence that the coenzyme fits in the active center of the apoenzyme in such a way that the pyrimidine moiety of the thiamine molecule stacks with the indole moiety of tryptophan in the active center of the protein (Kochetov et al. 1973; Aoki and Yamazaki 1980). Pyrimidine stacking occurs in thiamine-Cu-halogen complexes (Cramer et al. 1984; Archibong et al. 1989). Formation of thiamine - Cu complexes in-vitro has been described and analyzed by crystallography (Caira et al. 1974; Cramer et al. 1984; Archibong et al. 1989). However, such complexes have not been reported in-vivo.

The present study has shown that interactions among S, Mo, Cu and thiamine occur in the GI tract and systemically. Copper status was affected by excess Mo in the high S diet. The plausible mechanism is the formation of TM that subsequently affects Cu metabolism both in the GI and systemically. This effect appears to be specific for Cu. Copper status likely plays an important role in the metabolism of thiamine. Copper deficiency in ruminants could predispose an animal to thiamine deficiency, particularly in regions where animals are exposed to excess S in drinking water or feeds. Excess dietary Mo may exacerbate the effects of high S on Cu and thiamine status. Further studies are warranted in order to elucidate the mechanism by which these nutrients interact.

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### CHAPTER III. IN VITRO STUDY

The effect of sulfate on thiamine destroying activity in rumen content cultures in-vitro. A.A., Olkowski, B., Laarveld, J.F., Patience, S.I., Francis, and D.A., Christensen.

## THE EFFECT OF SULFATE ON THIAMINE-DESTROYING ACTIVITY IN RUMEN CONTENT CULTURES IN-VITRO

A.A. Olkowski, B. Laarveld, J.F. Patience, S.I. Francis, and D.A. Christensen.

### SUMMARY

The effect of sulphur on thiamine-destroying activity was studied in native or thiamine spiked rumen content cultures. The treatments consisted of water [control (Ctrl)], sodium chloride (NaCl) and sodium sulfate ( $\text{Na}_2\text{SO}_4$ ). A net gain (relative to 0 h) of thiamine in native cultures was observed for at least 48 h at levels of S ranging from 0.25 to 8 mg S added per ml of the culture and also in the Ctrl and respective NaCl cultures. However, after 48 h of incubation thiamine concentration declined ( $p < 0.05$ ) in native cultures treated with  $\text{Na}_2\text{SO}_4$  relative to Ctrl and NaCl treatments. There was a linear ( $p < 0.03$ ) time trend indicating an effect of  $\text{Na}_2\text{SO}_4$  over time on changes in thiamine concentration in native rumen cultures. In thiamine-spiked rumen content cultures, the cultures treated with  $\text{Na}_2\text{SO}_4$  produced higher ( $p < 0.05$ ) thiamine-destroying activity. There was no effect of cation (Na) on thiamine-destroying activity. Initial heat treatment of thiamine-spiked cultures inactivated part of thiamine-destroying activity. There were no differences among treatments in heat treated cultures. It is concluded that sulphate increases thiamine-destroying activity in the rumen content and the destructive mechanism involves a thermolabile factor(s), however, the ruminal synthesis of thiamine is not affected by sulphate.

## INTRODUCTION

Recent reports have indicated detrimental effects of excess dietary sulphur (S)<sup>1</sup> on thiamine metabolism in ruminants (Goetsch & Owens, 1987; Olkowski et al. 1991a). An epidemiologic study showed an increased occurrence of polioencephalomalacia (PEM) in cattle fed a high S ration (Raisbeck, 1982). Polioencephalomalacia is a central nervous system disorder which is commonly attributed to alterations in thiamine metabolism (Blood & Radostits, 1989), although the exact role of thiamine in the pathogenesis is not clear. While not specific, a convincing argument that some form of thiamine inadequacy may be indeed involved in the pathogenesis of the spontaneous cases of PEM was derived from observation that thiamine is an effective treatment. In most instances, thiamine administered in early stages of the disease leads to a complete clinical recovery (Blood & Radostits, 1989).

The disease was reproduced experimentally in sheep (Gooneratne et al. 1989b) and in cattle (Sadler et al. 1983; Gould et al. 1991) by feeding excess S. It was also observed incidently in nutritional trials involving dietary S (Khan et al. 1987; Gibson et al. 1988) and in cattle drinking high sulfate-water (Harries, 1987; Gooneratne et al. 1989a; Beke & Hironaka, 1991). The S induced PEM appears to

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<sup>1</sup> Sulphur (S) is used to denote a variety of sulphurous compounds since in ruminants the metabolism of excess dietary sulphur is cyclical, whereby all forms of dietary sulphur are reduced to sulphide and the absorbed sulphide is oxidized in the tissues to sulphate and recycled to the rumen.

be responsive to thiamine therapy (Harries, 1987; Khan et al. 1987; Gooneratne et al. 1989a).

Sulphur (organic and inorganic) metabolism in ruminants is unique in that it is largely influenced by ruminal microbes. Sulfate reducing bacteria have been isolated from the rumen of sheep (Huisinigh et al. 1974; Howard & Hungate, 1976). In the rumen, a variety of S-containing compounds are reduced to sulphite and sulphide (Lewis, 1954). Since the sulphite ions are known to destroy the thiamine molecule (Leichter & Joslyn, 1969), the pathogenesis of S induced PEM may be related to S and thiamine interactions in the rumen. Further, lower concentrations of thiamine in blood were seen in beef cattle drinking water containing high levels of sulfate (Gooneratne et al. 1989a; Olkowski et al. 1991a). However, it still remains to be determined whether the primary disturbances in thiamine metabolism associated with consumption of excess S are of ruminal origin. The objective of the present work was to study the apparent role of inorganic sulfate in thiamine-destroying activity in the rumen *in vitro*.

## **MATERIALS AND METHODS**

### **Animals**

Rumen contents were obtained from three mature cattle (two nonlactating cows and a steer) fitted with rumen cannulae. The animals were fed a maintenance ration (NRC-NAS 1984), consisting of brome hay (6-8 kg), dehydrated alfalfa pellets (1 kg) and concentrate-mineral mix (0.5 kg) and had free access to tap water. The dietary levels of S were 0.15% in feed DM and 23.5 ppm in water.

Care of animals was administered in accordance with the guidelines presented by the Canadian Council on Animal Care (1980).

### **Treatments**

The *in vitro* treatments were: deionized water [control (Ctrl)], sodium chloride (NaCl) and sodium sulfate ( $\text{Na}_2\text{SO}_4$ ). The NaCl treatment was included to consider a potentially confounding effect of cation (Na). Hence, the NaCl treatments contained the same amount of Na present in respective  $\text{Na}_2\text{SO}_4$  treatments. The amounts of S or Na added per ml of rumen content cultures used in various parts of the present study were either 0, 0.063, 0.125, 0.25, 0.5, 1, 2, 4 and 8 mg S or 0, 0.36, 0.72, 1.44, 2.87, 5.74 and 11.48 mg Na.

### **Rumen content cultures**

All materials and equipment used in these experiments were autoclaved at 121°C for 30 min and procedures were carried out using sterile techniques. Unless otherwise specified, the procedures were performed at room temperature.

In this study, we introduced a new procedure for *in vitro* study of rumen cultures. The nutritional base of our cultures was the diet containing all required nutrients. In order to simulate rumen content consistency the cultures were prepared to contain particulate matter (50 to 60% of total volume) and the liquid component containing soluble dietary nutrients and natural components of buffering system.

Rumen contents were harvested 2 to 3 h after the morning meal. The material was obtained from approximately 5 to 10 cm below the top layer. The

rumen content was gently squeezed by hand and the juice was collected into a glass beaker. The beaker was covered with aluminum foil and left undisturbed for 10 to 15 min to allow separation of liquid and particulate phases. For the best performance of the cultures, it was important to process rumen contents rapidly and with as little disturbance as possible. The floating particulate phase was collected and immediately distributed in 2.7 ml aliquots to culture tubes containing 300  $\mu$ l of a treatment (water, NaCl, Na<sub>2</sub>SO<sub>4</sub>) at required concentrations to provide a final volume of 3 ml. Thiamine was added to the thiamine spiked cultures at the rate of 50  $\mu$ g thiamine-HCl per culture tube. The tubes with cultures were gassed with 100% CO<sub>2</sub> and immediately sealed with air-tight rubber stoppers. In order to further ensure an anaerobic environment and provide room for gaseous products of microbial fermentation in the culture tubes, a part of the gas phase from tubes was removed with a syringe. The cultures were incubated in a water bath at 38°C while gently mixing. During the incubation, the cultures were inspected for overt fermentation activity several times daily. At the end of the respective incubation periods, the cultures were examined microscopically to evaluate the viability of microorganisms.

### **Experiments**

The effects of the treatments were studied in native rumen contents and thiamine-spiked rumen contents. The levels of S and the length of incubation periods required were determined in preliminary trials. Replicates of each treatment were prepared to provide identical sets for each incubation time. Sets of tubes from

each treatment were removed after predefined periods (see later) of incubation and were stored at  $-20^{\circ}\text{C}$  until analyzed.

Exp.1. The effects of  $\text{Na}_2\text{SO}_4$  added at the rate of 0, 0.25, 0.5, 1, 2, 4, and 8 mg S per ml of culture were studied in the native rumen contents. The respective NaCl treatments contained an equivalence of Na present in  $\text{Na}_2\text{SO}_4$  treatments. The measurements of thiamine were performed after 0, 12, 24, 48 and 72 h of incubation.

Exp.2. The effects of  $\text{Na}_2\text{SO}_4$  added at the rate of 0 or 0.25 mg S per ml of culture were studied in the thiamine-spiked rumen contents. The respective NaCl treatments contained an equivalent of Na present in  $\text{Na}_2\text{SO}_4$  treatments. The measurements of thiamine were performed after 0, 24, 48 and 72 h of incubation.

Exp.3. The response to increasing concentrations of sulfate (0, 0.063, 0.125, 0.25, 0.5, 1, and 2 mg S), arranged with 2.87 or 5.74 mg Na (as NaCl) added per ml of the culture was studied in the thiamine-spiked rumen contents. The measurements of thiamine were performed after 72 h of incubation.

Exp.4. The thermostable thiamine-destroying activity was studied in thiamine-spiked rumen contents with 1 mg S added per ml of culture. Sets in duplicate of each treatment were prepared and the cultures were autoclaved at  $121^{\circ}\text{C}$  for 30 min. The cultures were cooled down to room temperature and, after addition of thiamine, all tubes were processed as described above. One set was incubated for 72 h.

### **Thiamine analyses**

Thiamine contents in the cultures were analyzed using both a biological method

(Olkowski & Gooneratne 1991) and an HPLC method with post column derivatization.

Preparation of sample for analysis: For HPLC analysis, thiamine from the rumen contents was extracted by mild acid digestion using 0.05 M HCl (analytical grade). The rumen contents were mixed with acid at a ratio 1:4 v/v and incubated at 37°C for 30 min. The digest then was centrifuged for 15 min at 1000xg. The supernatant was evaporated under vacuum and the remaining residue reconstituted in an equal volume of the mobile phase (see later). The prepared sample was further diluted (as required) in mobile phase, filtered through a 0.45 µm membrane filter and injected into the column. For bioassays, thiamine was extracted as described by Olkowski & Gooneratne (1991).

Chromatographic analysis: For HPLC analysis, a reversed phase analytical column, (C<sub>18</sub>; 10 µm particle size, 250 X 4.1 mm) and a corresponding guard column (Phenomenex, Rancho Polos Verdas, CA) were used. The mobile phase was prepared from 0.05 M citrate buffer adjusted with aqueous 5 M KOH to pH 5.0 to which HPLC grade methanol was added to obtain a final concentration of methanol 8.2% v/v. The mobile phase was filtered through a 0.45 µm membrane filter. The mobile phase was delivered by a binary pump (Model 250, Perkin Elmer, Norwalk, CT) at a rate of 1 ml/min. The sample was introduced into the column by a sample injection valve (Model 210, Beckman Instruments, Fullerton, CA). The oxidizing reagent was K<sub>3</sub>Fe(CN)<sub>6</sub> (2.5 mM) dissolved in aqueous NaOH (3 M). The reagent was kept in a dark glass bottle at room temp. The oxidizing

reagent was delivered at a rate of 0.1 ml/min by an isocratic pump (Model 110A, Beckman Instruments, Fullerton, CA). The post-column reactor consisted of a PEEK zero-dead volume tee-piece (Upchurch Scientific, Oak Harbour, WA) and a teflon capillary 100 cm long wound to form a mixing coil. Detection was performed using a fluorescence detector fitted with 365 nm excitation and 470 nm emission filters (Spectrovision FD-100, Chelmsford, MD). The signal was displayed on a recorder (BD 41, Kipp & Zonen, The Netherlands).

### **Statistics**

Statistical analysis was carried out using the General Linear Model procedures of SAS (SAS Institute 1990). Repeated measures analysis of variance was used to identify main effects and interactions. Polynomial contrasts were used to identify time trends (Snedecor & Cochran 1989). Time-treatment interactions and time trends were further examined using contrasts. The contrasts of interest were: Ctrl vs NaCl, Ctrl vs Na<sub>2</sub>SO<sub>4</sub>, NaCl vs Na<sub>2</sub>SO<sub>4</sub>.

## **RESULTS**

### **Procedural**

**The cultures:** The technique for culturing rumen contents used in the present experiments yielded cultures with normal physiological parameters. The initial pH values (ranging 6.2 to 6.6) differed between individual animals but were not affected by the treatments. The pH values decreased gradually during the incubation periods, at 72 h ranging from 5.8 to 6.3. The control cultures (water) tended to have pH values slightly lower than NaCl or Na<sub>2</sub>SO<sub>4</sub> treated cultures.

The cultures had a viable protozoal population (microscopic evaluation) for at least 48 h and a viable bacterial population (microscopic evaluation and observable evidence of fermentation) for at least 72 h. However, high concentrations of  $\text{Na}_2\text{SO}_4$  tended to affect the viability of protozoa population. The 72 h incubation period used in the present study was chosen to ensure a comfortable survival of the cultures. After 72 h of incubation some 25-30% of undigested forage particles remained in the cultures. Almost all forage particles were digested after 96 h of incubation. At any interval of incubation, the viable cultures were clearly distinguishable from those which were not viable.

**The HPLC method:** The detection limit (baseline noise  $\times$  2) was 3 ng thiamine/ml. The method was validated using reproducibility and recovery tests. The recovery of thiamine from samples spiked with thiamine ranged from 95 - 110% and the reproducibility test showed coefficients of variation of less than 8%. The recovery of thiamine was not affected by the treatments. Standard curves using data points from five concentrations ranging from 10 to 160 ng/ml routinely had correlation coefficients exceeding 0.999.

## **Experimental**

**Exp. 1.** Increasing concentrations of S affected the survival of the protozoal population. Protozoa tended to be extinct within 3 h in cultures with 4 mg S added per ml of rumen culture. However, S at all concentrations did not appear to affect the viability of bacterial population. A net gain (relative to that at 0 h) of thiamine in native cultures was observed for at least 48 h at concentrations of

S ranging from 0.25 to 8 mg S added per ml of the culture and in respective NaCl and control treatments. There were within and between animal day-to-day variations with respect to thiamine concentration in the rumen content at 0 h, as well as in the magnitude of the net gain of thiamine in the cultures, although all trials gave qualitatively similar results. Thiamine synthesis was similar among treatments during the first 24 h (Fig.1). At 48 h thiamine content in Na<sub>2</sub>SO<sub>4</sub> cultures was lower than in Ctrl and NaCl cultures. At 72 h of incubation thiamine concentration declined in all treatments but the decrease was more pronounced in Na<sub>2</sub>SO<sub>4</sub> treated cultures. There was a time effect ( $p < 0.0001$ ) with time x treatment interaction contrasts being: NaCl vs Ctrl ( $p = 0.86$ ), NaCl vs Na<sub>2</sub>SO<sub>4</sub> and Ctrl vs Na<sub>2</sub>SO<sub>4</sub> ( $p < 0.045$ ). There was a linear ( $p < 0.03$ ) time trend, with treatment differences: NaCl vs Ctrl ( $p = 0.72$ ), NaCl vs Na<sub>2</sub>SO<sub>4</sub> ( $p < 0.02$ ) and Ctrl vs Na<sub>2</sub>SO<sub>4</sub> ( $p < 0.028$ ), indicating an effect of S over time on changes in thiamine concentration in native rumen cultures.

**Exp. 2.** Changes in thiamine content in thiamine-spiked rumen content cultures are shown in Fig.2. There were similar ( $p = 0.22$ ) thiamine destroying activities in Ctrl and NaCl cultures. However, the Na<sub>2</sub>SO<sub>4</sub> cultures had higher thiamine destroying activity relative to Ctrl ( $p < 0.004$ ) and NaCl ( $p < 0.002$ ). Polynomial contrasts analyses revealed a linear time trend ( $p < 0.009$ ) for treatment effects with contrasts between treatments being: Ctrl vs NaCl ( $p = 0.1$ ), Ctrl vs Na<sub>2</sub>SO<sub>4</sub> ( $p < 0.017$ ) and NaCl vs Na<sub>2</sub>SO<sub>4</sub> ( $p < 0.004$ ). Substantial differences in thiamine concentrations were observed in S treated cultures when the results obtained using the biological

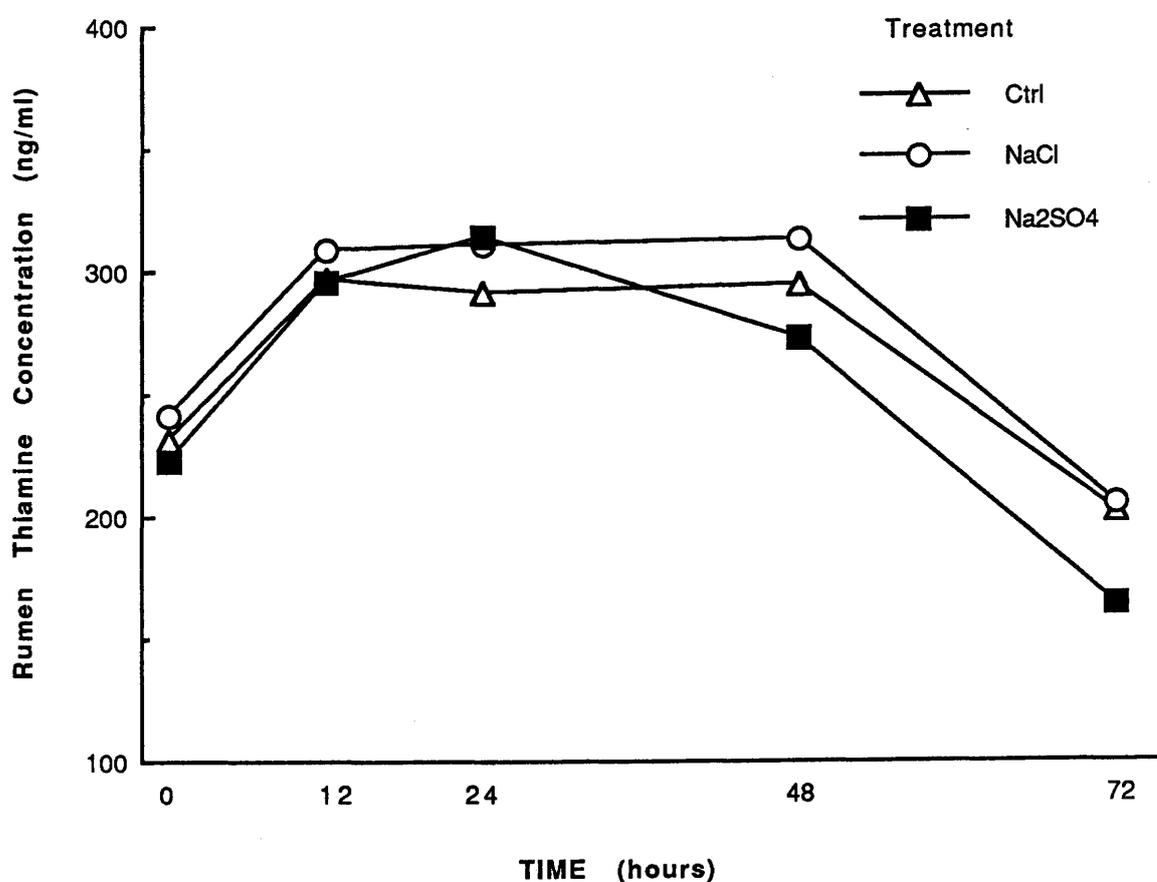


Fig. 1. Thiamine concentration in native rumen content cultures treated with water (Ctrl), NaCl and Na<sub>2</sub>SO<sub>4</sub>. The data represents means of two observations where sulphate was added at a rate of 1 or 2 mg S per ml rumen content culture. The NaCl treatments contained Na equivalent to the amount of Na present in respective Na<sub>2</sub>SO<sub>4</sub> treatments. Pooled standard errors for hours 0, 12, 24, 48, and 72 were 17.6, 19.1, 19.4, 16.2, and 26.7, respectively. The thiamine-destroying activity was higher ( $p < 0.05$ ) in sulfate treated cultures.

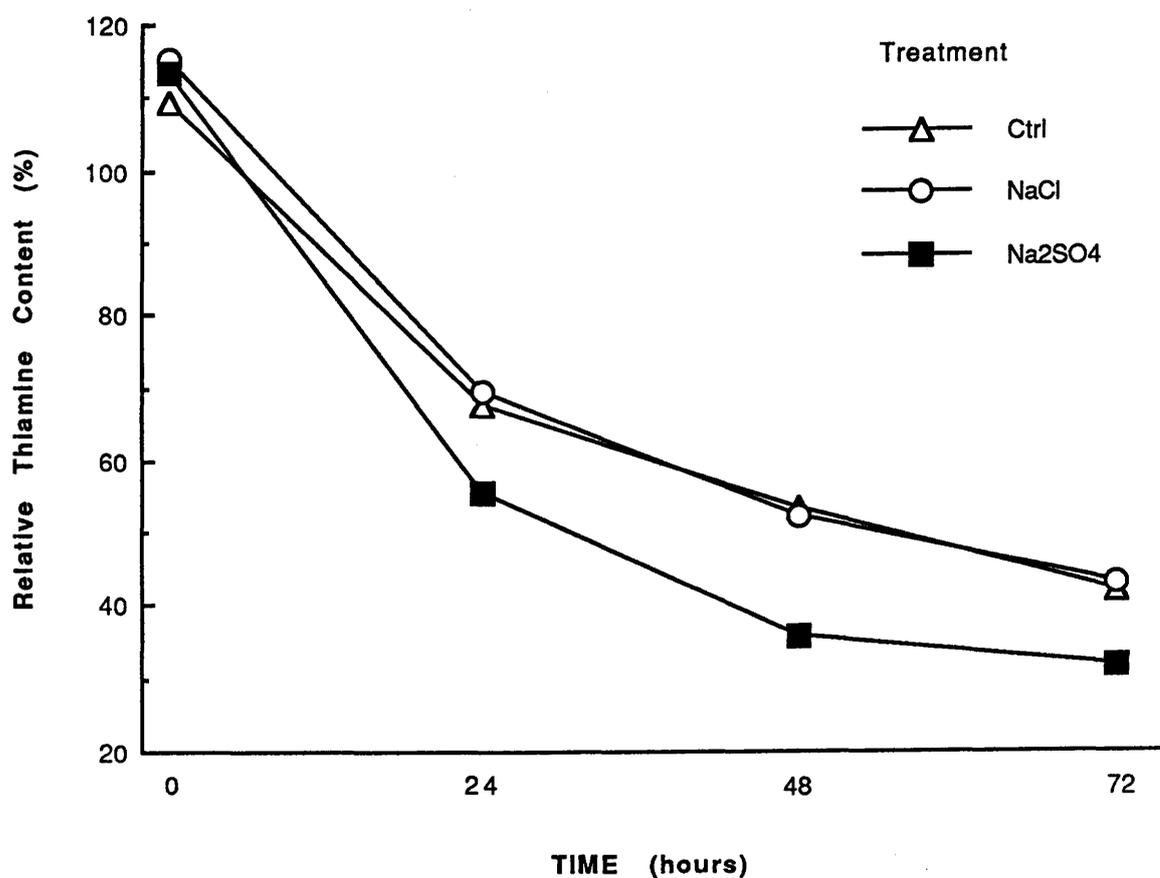


Fig. 2. Thiamine-destroying activity in thiamine spiked rumen content cultures treated with water (Ctrl), NaCl and Na<sub>2</sub>SO<sub>4</sub>. Sulphate was added at a rate of 0.25 mg S per ml rumen content cultures. The NaCl treatments contained Na equivalent to the amount of Na present in respective Na<sub>2</sub>SO<sub>4</sub> treatments. Pooled standard errors for hours 0, 24, 48, and 72 were 1.6, 2.9, 4.5, and 4.2 respectively. The Na<sub>2</sub>SO<sub>4</sub> treated cultures had higher thiamine-destroying activity than Ctrl ( $p < 0.004$ ) or NaCl ( $p < 0.002$ ).

method for thiamine determination were contrasted with the results obtained using the HPLC method. Fig. 3 shows a comparison of thiamine remaining in thiamine-spiked cultures after 72 h of incubation as detected by HPLC or the biological method. During the first 24 h of incubation both methods of measurement showed a similar trend of thiamine destroying activity (data not shown). However, in cultures incubated for 72 h, the biological method detected approximately 10 to 25% less thiamine in NaCl or Ctrl cultures, and 35 to 50% less thiamine in Na<sub>2</sub>SO<sub>4</sub> treated cultures.

**Exp. 3.** There was an overall effect of S concentration on thiamine-destroying activity ( $p < 0.026$ ) but it was not influenced ( $p = 0.68$ ) by the level of cation (Fig.4). The increment in activity in response to increasing concentrations of S was quite small. Under these experimental conditions, the thiamine destroying activity appeared to reach a plateau level (based on individual animal data) at approximately 0.25 to 1.0 mg S added per ml rumen content culture. There were between animal variations with respect to responses to increasing concentration of S. Further increases of S did not enhance thiamine-destroying activity.

**Exp. 4.** In the heat treated cultures, thiamine-destroying activities were lower ( $81.9\% \pm 3.8$  (SE) of initial thiamine remained after 72 h of incubation) than in non-autoclaved cultures. There were no differences among treatments. The thermostable thiamine destroying activity accounted for approximately 20-40% of total background activity (35-60%) seen in non-autoclaved Ctrl and NaCl cultures.

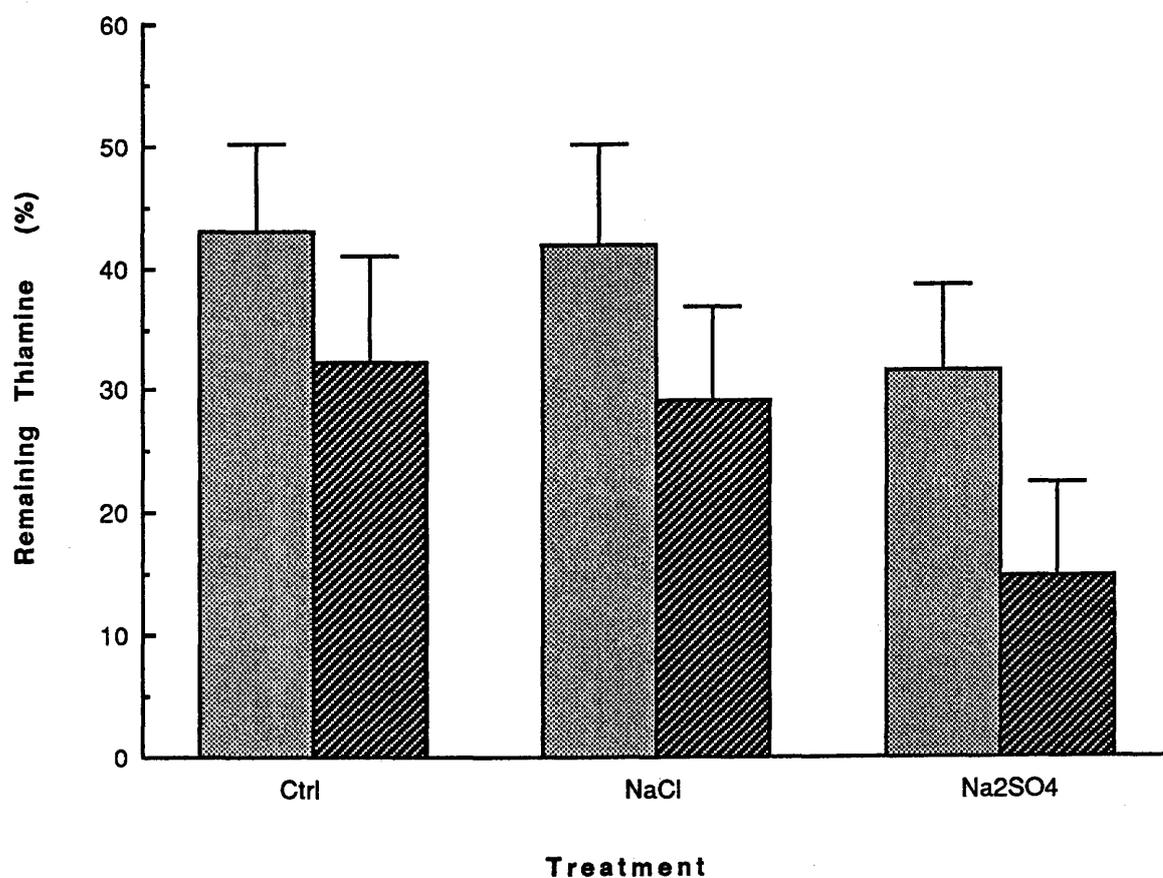


Fig. 3. Contrast in remaining thiamine, as detected by HPLC and biological methods, in rumen content cultures treated with water (Ctrl), NaCl and Na<sub>2</sub>SO<sub>4</sub> after 72 h of incubation. Sulfate was added at a rate of 0.25 mg S per ml rumen content cultures. The biological method detected approximately 10 to 25% less thiamine in NaCl or Ctrl cultures, and 35 to 50% less thiamine in Na<sub>2</sub>SO<sub>4</sub> treated cultures.

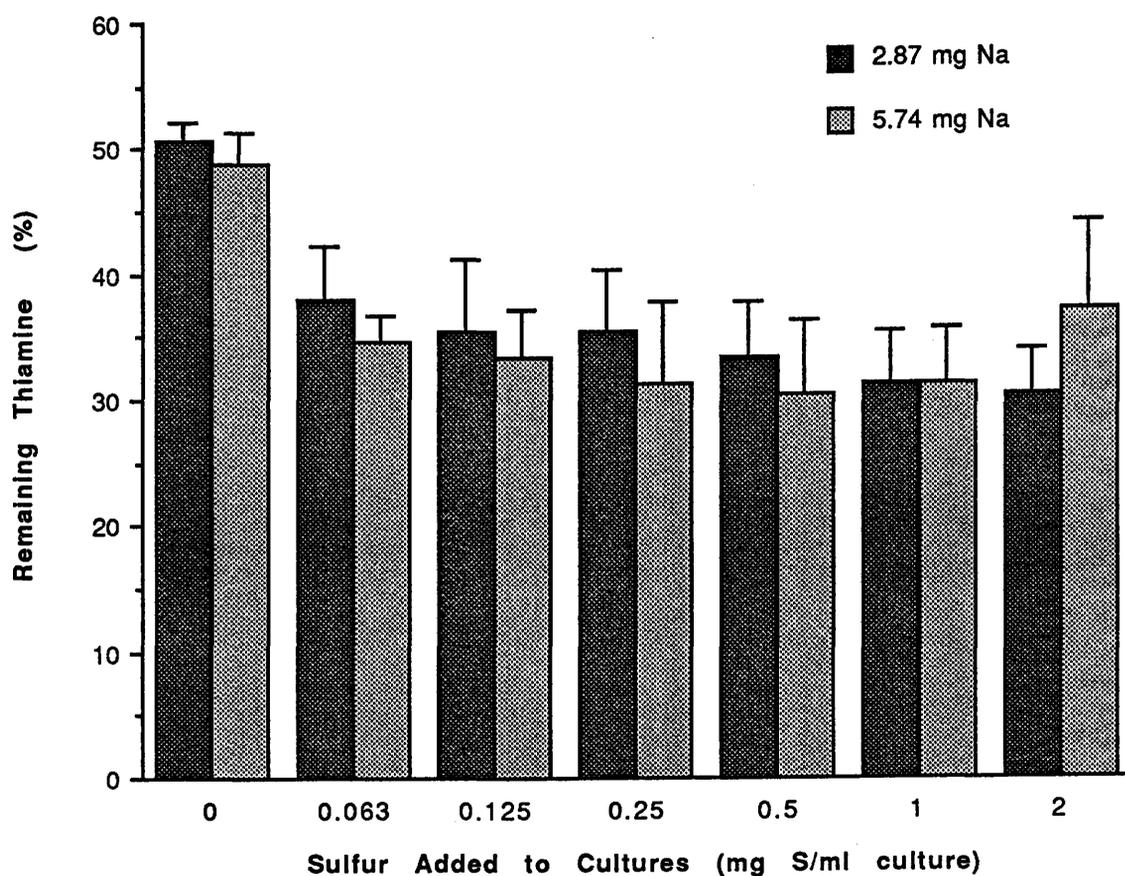


Fig. 4. Changes in thiamine-destroying activity in rumen content cultures treated with sulfate added at an increasing rate ranging from 0.063 to 2 mg S per ml rumen culture. Each concentration of S was matched with constant level of sodium ion (as NaCl) added at a rate of 2.87 or 5.74 mg Na per ml rumen content cultures. There was an overall effect ( $p < 0.026$ ) of S concentration and no effect ( $p = 0.68$ ) of cation (Na) on thiamine-destroying activity.

## DISCUSSION

The present experiments indicate that addition of sulfate ions has a destructive effect on thiamine in rumen cultures *in vitro*. In particular, this data demonstrates that the S effect is dependent on a thermolabile factor.

Notably, the environment of the forestomachs of ruminants appears to be quite "hostile" to thiamine. Under our experimental conditions, 40-60% of added thiamine was destroyed in control cultures. This is consistent with observations *in vivo* that less than 60% of ruminal thiamine enters the duodenum (Zinn et al. 1987; Goetsch & Owens, 1987). Similarly the net gain of thiamine in our rumen content cultures is in accord with the occurrence of the synthesis of thiamine in the rumen *in vivo* reported by some authors (Zinn et al. 1987; Goetsch & Owens, 1987).

In the present study, the thiamine-destroying activity occurred in all cultures. This activity can be ascribed to at least two categories of factors i.e. thermostable and thermolabile. Thiamine-destroying activity in the rumen of cattle, attributed to a thermostable factor of small molecular weight, was described by Quaghebeur et al. (1975). On the other hand, in the study of Loew et al. (1970), the thiamine-destroying activities in the rumen content of normal and PEM cattle were mostly due to thermolabile factor, and very little thiamine-destroying activity occurred in autoclaved rumen content. Sulphur was not measured in these studies. In the present study, it appears that regardless of treatment, the bulk of thiamine-destroying activity in the rumen content was a consequence of the thermolabile

activity. It is notable that there was no effect of sulphate on thiamine destruction in autoclaved cultures. Hence, the effect of sulphate appears to be linked to thermolabile activity. The reduction of sulphate to sulphite by rumen bacteria (Lewis, 1954) and the subsequent destruction of thiamine molecules by sulphite ions (Leichter & Joslyn, 1969) may explain the molecular basis of S-induced thiamine-destroying activity in the rumen.

Raisbeck (1982) hypothesized that destruction of thiamine in the rumen by sulphite, which is the product of microbial sulphate reduction, may be the cause of S induced PEM. The present study shows that S caused an increase in thiamine-destroying activity in the rumen. Furthermore, the involvement of sulphite ions in the thiamine-destroying activity in the rumen appears to be a very plausible assumption considering the chemistry of thiamine-sulphite interactions. Notably, the preferable conditions for the reaction of sulphite and thiamine, i.e. anaerobic and nitrogen rich environment (Kawasaki et al. 1958) and a pH of 5.5 to 6.5 (Leichter & Joslyn, 1969) are all present in the rumen.

It appears that some aspects of rumen bacterial activity are little affected by S. Kennedy et al. (1971) found no depression of starch digestion by rumen microorganisms when up to 11 mg S per ml rumen content was added. Similarly, it appears that  $\text{Na}_2\text{SO}_4$  per se does not affect thiamine synthesis in the rumen to any significant extent. In the present study, a net gain (relative to 0 h) of thiamine in native cultures treated with  $\text{Na}_2\text{SO}_4$  added at a rate as high as 8 mg S/ml rumen content was observed during the first 48 h of incubation. Somewhat

different profiles were seen in thiamine-spiked cultures, where the detrimental effect of S was apparent at 24 h. However, it must be stressed that in native cultures the bulk of thiamine is mainly in the intracellular compartment. From our experience, it appears that approximately 10 to 20% of total rumen thiamine is distributed in the cell free fraction of the rumen juice (unpublished observations). In the study of Quaghebeur et al. (1975), free thiamine constituted 5 to 20% of total thiamine. Apparently, the gain of thiamine observed in our cultures during the first 48 h was a net difference between thiamine synthesised and destroyed, since the disappearance of thiamine from the cell free fractions was notable after 24 h (unpublished observation). Likely, the intracellular thiamine was more protected from the detrimental effects of S, however, as the digestion progressed the thiamine was released from the cells and exposed to destruction. It is noteworthy that in the thiamine-spiked cultures, where all added thiamine remained extracellular, the destroying activity was apparent within the first 24 h.

In the present study, addition of  $\text{Na}_2\text{SO}_4$  to the cultures resulted in a 15 to 25% increase above background of thiamine-destroying activity. The significance of these findings to the *in vivo* situation is yet to be evaluated. Under experimental conditions, dietary sulfate supplementation tended to reduce duodenal flow of thiamine in cattle, although a decrease in plasma thiamine was not apparent (Goetsch & Owens, 1987). However, in a recent field study (Olkowski et al. 1991a), 20% of a population of cattle drinking high sulfate water

had blood thiamine concentrations indicative of marginal deficiency. In vivo, the effect of S on the overall thiamine status may be modest if the content of thiamine in the feed is high or when the ruminal synthesis is extensive. However, should any of the above factors be inadequate, the S may have a physiologically significant effect. Furthermore, the detrimental effect of dietary sulfate on thiamine may be more pronounced in vivo, since the population of sulfate reducing bacteria in the rumen increases when animals are fed excess dietary S (Lewis, 1954). Also, the effect of S may be more harmful under some dietary regimes. A net loss of thiamine was observed when high concentrate diets were fed (Miller et al. 1986). Interestingly, PEM in cattle is most often associated with the feeding of a high concentrate diet, and sudden replacement of roughage with grain (McGuirk, 1987). The described instances of S induced PEM were in cattle under feedlot conditions (Raisbeck, 1982; Sadler et al. 1983; Khan et al. 1987, Harries, 1987; Gooneratne et al. 1989a).

Notably relatively less thiamine was detectable by the biological method than by the HPLC method particularly in the  $\text{Na}_2\text{SO}_4$  treated cultures. This may indicate that the addition of S to the cultures created an environment in which the bioavailability of thiamine was affected. In the context of these findings, perhaps the fate of remnants of destroyed thiamine molecules deserves more attention. In vitro, sulphite ions not only cleave thiamine molecules into pyrimidine and thiazole moieties but also can act as a catalyst in nucleophilic substitution reactions (Zoltewicz et al. 1982) . The substitution is similar to the enzymatic

reaction of thiaminease I where the thiazole moiety can be substituted by another heterocyclic compound. A number of possible substrates for this reaction may be commonly found in the rumen content (Roberts & Boyd 1974, Brent & Bartley, 1984) and the product can be a thiamine-like compound, possibly having antimetabolite properties.

In summary, it is concluded from these studies that addition of sulphate has a detrimental effect on thiamine metabolism in ruminants. One of the possible sites of thiamine-destroying activity is the rumen. The cause of sulphate-induced reduction in duodenal flow of thiamine observed *in vivo* (Goetsch & Owens, 1987) is likely destruction of thiamine, since ruminal synthesis of thiamine appears to be unaffected by sulphate. The sulphate induced thiamine-destroying activity in the rumen is dependent on a thermolabile factor(s). Hypothetically, the sulphate induced deterioration of thiamine in rumen content can be attributed to sulphite ions, the product of bacterial reduction of sulphate.

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**CHAPTER IV.**  
**GENERAL DISCUSSION AND CONCLUSIONS**

## GENERAL DISCUSSION

### 1.1 Summary of pertinent facts

The effects of excess dietary S in ruminants have discrete features not seen in other farm animals. The best investigated is the effect of S on Cu metabolism. However, the work presented in this thesis shows that the spectrum of toxic effects is much broader. It became clear that thiamine metabolism is also affected by S, and more complex three way interactions of S-Cu-thiamine may be involved.

In the past, the effect of S on Cu metabolism was examined mostly in the context of Cu deficiency. However, the toxic effect of dietary S could not invariably be attributed to Cu depletion (Smart et al. 1986). The harmful effects of dietary S on thiamine status of cattle was noted in our first field study (Gooneratne et al. 1989a). The next field studies which included a large number of farms in Saskatchewan (Olkowski et al. 1991a) showed that cattle exposed to high S levels in drinking water had blood thiamine levels lower than those in cattle drinking water containing a low concentration of S. These studies also concluded that a subpopulation of exposed animals was thiamine deficient. However, the following field investigation conducted on a herd of cattle where the outbreak of PEM was attributed to high S in drinking water did not indicate thiamine deficiency per se. The occurrence of PEM was indeed documented in all of our field research, but it became obvious that simple thiamine deficiency is

not an etiological factor in S induced PEM. A similar conclusion was derived from the controlled study in vivo (Olkowski et al. 1992).

The findings presented in this thesis indicate that S induced PEM does not fit the model of the condition induced by dietary thiamine deprivation (Butterworth et al. 1985, Giguere and Butterworth 1987). However, thiamine appears to be an effective treatment (Harries 1987, Gooneratne et al. 1989a, Olkowski et al. 1992, Olkowski and Christensen 1992) in S induced PEM, but no evidence was found in our experiments that overt thiamine deficiency occurred in clinically affected animals.

Interestingly, the evidence of metabolic thiamine deficiency was found in apparently healthy young and adult cattle (see Chapter I). This indicates that thiamine deficit cannot be correlated with clinical events. However, thiamine treatment can be associated with recovery. The curative effect of thiamine in PEM may have some pharmacological features.

It appears that thiamine is involved in some, unrecognized from a molecular standpoint, metabolic events in S induced PEM. Dietary thiamine supplementation in sheep fed high S diets did prevent manifestation of clinical signs and gross brain lesions, but did not prevent microscopic lesions (Rousseaux et al. 1991). The early and/or subclinical metabolic lesions were also evident as impaired electrophysiological events along the auditory pathway (Olkowski et al. 1990a).

Our studies confirmed that S affects Cu metabolism and that the detrimental effects of S on Cu metabolism could be alleviated by providing Cu supplements. Further, it was also observed that Cu influences interactions between S and thiamine. Both Cu and thiamine depletion in the blood of cattle drinking water containing a high concentration of S was corrected by dietary supplementation of Cu alone (Gooneratne et al. 1989a). The interaction of S-Cu-thiamine was subsequently studied in more detail (Olkowski et al. 1991b), showing that the interaction among these nutrients occurs in the GI tract as well as systemically.

Notably, S was used to induce Cu deficiency in numerous studies on the immune system response to Cu deficiency in ruminants, but thiamine metabolism was not considered in these studies. Our study showed that thiamine may play an essential role in such S induced problems. Impairment of the neutrophil function was attributed to an apparent subclinical thiamine deficiency (Olkowski et al. 1990b).

During the course of this study, several novel metabolic events were noted. Some of the basic interpretations were provided on the basis of the results, but more detailed studies would be desired. Thus, some of the observations documented in the present thesis cannot be explained at this moment with the support of tangible experimental evidence. Possible hypothetical deliberations and/or supportive evidence of work from other workers published after the completion of our experiments will be presented in the following sections.

## **2.1 Overall Analysis of the Findings.**

### **2.1.1 Alteration of electrophysiological events in the brain.**

We used the technique of evoked potentials to evaluate the effect of excess dietary S on early subclinical changes along the auditory pathway (Olkowski et al. 1990a). This technique allowed us to detect alterations in electrophysiological patterns which can be attributed to S. Some of the changes were reflected by the presence of histological lesions in pertinent areas of the brain stem. This approach has yet to be evaluated by others. However, since the completion of our study a similar idea to study brain electrophysiology of PEM affected animals was utilized by Strain et al. (1990). However, the latter authors, unlike us, used visual evoked potentials. The value of these means as a diagnostic tool for the early recognition of PEM remains to be verified by more clinical trials.

### **2.1.2 Sulfur effect on neutrophil function.**

In one of our trials (not presented in this thesis) we tried to explain observed S induced impairment of neutrophil function in vitro using the concept of S induced subclinical deficiency of thiamine with respect to energy metabolism, hexose monophosphate pathway, and scavenging of free radicals. Since our study was published (Olkowski et al. 1990b) other research teams reported results which could provide some support for our hypothesis. Khan et al. (1990) reported a reduction in zymosan-stimulated respiratory rates of pulmonary alveolar macrophages collected from rats exposed to hydrogen sulfide. Ozawa and Hanaki (1990) suggested formation of thiosulfate and sulfide radical anions from

the reactions of hydrogen peroxide with thiosulphate and sulphide ions respectively. They suggested that these radical anions may be important in the toxicity of the sulfide ion in living cells. The involvement of free radicals in S toxicity is supported by the earlier work of Mottley and Mason (1988) who showed that sulfite derived radicals can be formed through the catalytic reaction of peroxidases. Another interesting development was the finding that sulfide has a pronounced effect on the complement component C3 in the form of C3bi. Granlund et al. (1991) observed that in vitro, sulfide releases the C-terminal region of the alpha chain from C3bi. The latter authors further speculated that if sulfide has the same effect on C3bi deposited on the bacterial surface as it has on C3bi in solution, it would annihilate the very important contribution of C3bi to opsonization. More work is needed to understand the mechanism of S effects on the immune system.

### **2.1.3 Sulfur effects on blood thiamine levels**

Observations on the effects of S on blood thiamine status made during various stages of this work brought about variable results (Gooneratne et al. 1989a, Olkowski et al. 1991a, Olkowski and Christensen 1992). Lowered blood thiamine concentrations were not consistently observed in PEM affected animals (Loew and Dunlop 1972b). This discrepancy was attributed to the nonspecific methods used for thiamine analysis reported in older literature (Loew and Dunlop 1972b, Rammell and Hill 1986). However, the thiamine measurements in the present study were performed using a highly sensitive biological method,

yet there was no consistent trend in changes in blood thiamine concentrations of animals exposed to high dietary S. Further, the animals showing clinical signs of PEM in our observations had consistently higher blood thiamine concentrations when compared to apparently normal non-affected animals (Gooneratne et al. 1989b, Olkowski and Christensen 1992).

An apparently important bearing on the level of blood thiamine in animals exposed to excess dietary S may be the initial level of thiamine present in the feed. The recently completed *in vitro* study (Chapter III) showed that addition of S to rumen content cultures increased thiamine-destroying activity, although in comparison to overall thiamine-destroying activity in the rumen, this effect of S appeared to be relatively small. Hence, it is possible that if the level of thiamine in the feed is high and/or ruminal synthesis is brisk, the blood thiamine concentration may not be affected by dietary S. However, should any of these conditions be compromised, the destruction of thiamine could be high enough to affect blood thiamine status. This explanation is in accord with the *in vivo* study, where some authors (Goetsch and Owens 1987, Zinn et al. 1987) observed net synthesis of thiamine in the rumen, whereas others (Millar et al. 1986) observed net loss of thiamine in the rumen. It is important to stress that blood thiamine was higher in sheep fed excess dietary S where the feed thiamine level was also relatively high (14 mg/kg) (Olkowski et al. 1992), and in the field investigation where cattle were grazing pasture (Olkowski and Christensen 1992). On the other hand two field studies, involving feedlot cattle (Gooneratne et al. 1989a, Olkowski

et al. 1991a), showed lower blood thiamine concentrations in animals exposed to high S water. Among those exposed to high S, but not affected clinically, several animals had blood thiamine concentration indicative of deficiency.

However, the problem appears to be more complex. The possibility of more (unknown) factors (other than S) affecting thiamine metabolism should be considered. In general, it appears that the environment of the forestomachs is not favorable to the preservation of thiamine molecules. At some point more than 40% of ruminal thiamine is destroyed before it enters the duodenum (Goetsch and Owens 1987, Zinn et al. 1987). This seems peculiar in a ruminant species which from an evolutionary point of view relied on ruminal synthesis of thiamine. However, it has to be stressed that a number of thiamine related problems occur mostly under conditions of intensive livestock production, where the metabolic adaptation acquired through evolution must deal with considerably distorted, unnatural dietary factors.

In the two field studies (Gooneratne et al. 1989a, Olkowski et al. 1991a) high levels of S can be clearly associated with lower blood thiamine concentration, but several animals drinking low-sulfur water also had low blood thiamine concentrations (Olkowski et al. 1991a). Hence, there is a possibility of the presence of other thiamine-destroying factors. Indeed, the bulk of thiamine-destroying activity in rumen cultures in vitro could not be attributed to the S treatment. An intriguing problem arises from the observation of an apparent elevation of blood thiamine concentration in animals affected with PEM

(Gooneratne et al. 1989b, Olkowski and Christensen 1992). This problem will be discussed later.

#### **2.1.4 Thiamine status and S induced PEM**

Whether or not thiamine status can be related to the development of PEM remains unresolved. Historically PEM has been regarded as an effect of thiamine deficiency (Daly 1968, Edwin et al. 1968, Loew and Dunlop 1972a, Edwin and Jackman 1973). The concept of thiamine deficiency as an etiological factor in PEM was mainly based on observations that affected animals were responding to treatment with thiamine since thiamine status was not always consistently low. In the majority of field cases of PEM (Pill 1967, Daly 1968, Loew 1975, Dickie et al. 1979, Thomas 1986, McGuirk 1987) as well as in the cases likely associated with excessive intake of S (Harries 1987, Gooneratne et al 1989a, Khan et al. 1987, Rousseaux et al. 1991), thiamine is an effective treatment. However, it is debatable whether thiamine deficiency per se is the causative factor. Or, is the disease simply responding to treatment with thiamine for other unknown reasons?

The present study added to the controversy of thiamine involvement in PEM. The nature of the lesions observed in this study fit the general features of lesions commonly seen in PEM (Sullivan 1985). However, some distinctive pathological features were found in the field cases of S induced PEM (Clark, personal communication, see Chapter I). In the described outbreak of PEM attributed to consumption of high sulfate-water, the disease occurred in mature animals,

whereas it more commonly affects younger stock (Sullivan 1985). Interestingly, the blood thiamine concentration was elevated at the onset of clinical signs of S induced PEM (Gooneratne et al. 1989b). Furthermore, the host thiamine status, on the basis of commonly accepted indices, did not indicate overt thiamine deficiency in animals exposed to high dietary S (Olkowski et al. 1992). Yet in the field cases thiamine was an effective treatment.

In the present study, an interesting observation was the lack of apparent clinical signs in sheep which had malacic lesions in the brain (Rousseaux et al. 1991). This indicates the existence of a subclinical form of this condition and opens the possibility that the disease may be more prevalent than previously thought. Furthermore, dietary thiamine supplementation prevented development of clinical signs and gross lesions, although it did not totally prevent the development of microscopic brain lesions. This observation further complicates the concept of overt thiamine deficiency as an etiological factor in PEM.

The reports on thiamine status in PEM are conflicting. The lack of consensus in can be partially attributed to a lack of a uniform approach to experimental models and standardized methodology and sampling protocol. However, the currently used approach does not provide evidence to distinguish whether the necrotic changes in the cell are due to the decline in TPP dependent enzymes activity, or if the decline in enzyme activity is because the brain tissue is dying of a different cause. Our experience indicates that samples taken from necrotic

areas of the brain would show decreased thiamine concentration and lowered enzymes activities.

It appears that in some instances of PEM, the structural damage may not always be reflected as a functional defect. The occurrence of brain lesions without apparent clinical signs in farm animals is not well documented. Prior to our study, only one study on thiamine deficiency in lambs reported very mild and localized brain lesions in animals killed before the development of clinical signs of PEM (Thornber et al. 1979). However, the phenomenon of absence of clinical signs even in the presence of extensive damage to the brain tissue is well documented in laboratory animals (Iwasaki et al. 1989) and humans (Wallis and Wilson 1983, Bonnell and Moskvina, 1985, Fazekas 1989).

One of the stronger pieces of evidence that thiamine deficiency is the etiological factor in PEM is derived from the observation that massive doses of thiamine can be an effective treatment. However, is the curative effect of thiamine exerted via the improvement of the activities of TPP dependent enzymes? It is notable that animals exposed to high dietary S, which did not show clinical signs of PEM at all or those which survived the initial onset of clinical signs and completely recovered, did have gross malacic lesions in their brains (Rousseaux et al. 1991). This suggests that the regeneration of existing lesions is not necessarily associated with recovery from clinical signs and that the presence of necrotic lesions is not necessarily associated with the clinical signs. Hence, the effect of thiamine as a panacea in PEM may be associated with the

alleviation of clinical signs (likely some pharmacological effect) rather than with the repairing of necrotic lesions.

#### **2.1.4.1 Effects of excess dietary S on thiamine metabolism.**

Excess S has a detrimental effect on thiamine metabolism in ruminants. The *in vitro* study (Chapter III) showed that one of the possible sites of thiamine destruction is the rumen. Similarly, Goetsch and Owens (1987) observed a reduced duodenal flow of thiamine, but this was not completely consistent with the lowering of plasma thiamine status. In an attempt to explain the pathogenesis of S induced PEM, Raisbeck (1982) hypothesized that the sulfite ion produced in the rumen may destroy ruminal thiamine. Our *in vitro* study showed that addition of S indeed increases thiamine-destroying activity in the rumen. However, the concept of massive destruction of thiamine in the rumen leading to systemic deficiency as the sole cause of S induced PEM is not supported by the data obtained in the present study. No conclusive evidence of either organ - localized or systemic thiamine deficiency *per se* was found in animals exposed to high dietary S (Olkowski et al. 1992, Olkowski and Christensen 1992), although a mild to moderate metabolic deficit of thiamine was apparent. Interestingly, the metabolic deficit of thiamine cannot be directly associated with the development of clinical signs of PEM (Olkowski and Christensen 1992). Undoubtedly, low thiamine status would increase the risk of PEM in animals exposed to high dietary S (Olkowski et al. 1992).

#### 2.1.4.2 Thiamine deficiency: Classical concept

Measurements of blood TK activities and TPP effects are commonly accepted as the best indices of thiamine status of animals (Bogin et al. 1985, Ali et al. 1987, Blood and Radostits 1989). However, can blood parameters be used to evaluate thiamine status of the brain?

Supportive evidence that the deficiency definition, based on reduced activity of thiamine-dependent enzymes, may not be valid comes from research with other species, since the information on the overall metabolism of thiamine in ruminants is sparse. It has been shown (Brin 1962, Pincus and Wells 1972) that the brain retains its thiamine content in the face of a thiamine-deficient diet with great tenacity and a decrease in activities of thiamine dependent enzymes does not precede the development of neurological signs. Indeed, on the basis of the studies on rats (Brin 1962, Pincus and Wells 1972) it is difficult to implicate a decreased activity of the TPP-dependent enzymes in nervous tissue as a primary biochemical event leading to neurological impairment. Also, a more recent study indicates that in pyriithiamine induced thiamine deficiency in rats, the selective cellular responses cannot be related to enzymatic alterations (Gibson et al. 1989). The latter authors concluded that factors other than decreases in activities of thiamine dependent enzymes must underline selective vulnerability of certain brain regions.

Interestingly, even using a perfect model of dietary thiamine deprivation in rat (Brin 1962, Pincus and Wells 1972, Butterworth et al.1985, Giguere and

Butterworth 1987), there is no clear association between thiamine deficiency and clinical signs. It must be emphasized that in response to dietary thiamine deprivation, signs of deficiency in the blood and other tissues are evident within 4 to 7 days (Brin 1962), but there is a lack of temporal decline in the brain's thiamine and TPP dependent enzymes activities in presymptomatic animals. In the typical deficiency situation one would expect a gradual decline in the vitamin concentration and enzymes activities, with some threshold triggering necrotic events in the cell.

Thus far, most of the effort to explain the events associated with thiamine deficiency was concentrated on TPP dependent enzymes and metabolic consequences of impaired function of these enzymes. The possibility that thiamine plays a role in alleviation of S induced lesions in the brain tissue that is independent of its coenzyme function deserves more attention.

#### **2.1.4.3 Thiamine deficiency and PEM: An alternative approach**

For years, researchers were trying to explain the structural damage seen in PEM, solely as an effect of an overall impaired function of thiamine dependent enzymes, and subsequent metabolic disorder mainly in energy metabolism. Many workers failed to show a direct link between the activity of these enzymes and clinical signs or morphological changes. Apparently, thiamine deficiency per se can account for development of PEM lesions in pre-ruminant animals (Thorner et al. 1979), but to reproduce such a situation in ruminating animals would be

virtually impossible. The study of Mueller and Asplund (1981) failed to show that dietary thiamine deprivation can produce PEM.

The present data indicate that the S induced disease cannot be explained solely by metabolic changes originating from depletion of thiamine. However, it is possible that in S toxicity, the actual structural damage may be an effect of oxidative damage to vital subcellular structures inflicted by S metabolites such as sulfides, sulfites and/or their more reactive derivatives. The possibility of synthesis of S derived free radicals (in the rumen and/or systemically) and their injurious potential is supported by the work of Mottley and Mason (1988) and Ozawa and Hanaki (1990).

It is noteworthy that the thiamine molecule may have natural features enabling protective function against S metabolites. Free thiamine may offer protection by scavenging free radicals (Theron et al. 1981). Also, thiamine in its thiazole open form with the exposed -SH group could serve as a conjugate to detoxify sulfide derivatives. Further, the readiness of thiamine to react with sulfite ions (Leichter and Joslyn 1969) may indeed be considered a detoxifying process. These processes would be at the expense of destroying the thiamine molecule, with possible consequences of secondary metabolic deficit. Hence, the hypothesis of increased requirement for free thiamine (independent from the requirement of TPP dependent enzymes) in S toxicity seems plausible.

The increased metabolic demand for thiamine may be a factor in the pathogenesis of PEM. Sager et al. (1990) did not observe changes in TK and TPP

effect values in PEM affected calves, yet the animals responded to parenteral treatment with thiamine. Thus, it appears that the therapeutic effect of thiamine may be other than supplying the TPP co-factor for enzymatic activity, i.e. thiamine may have some unknown "protective/sparing" effects. This is consistent with previous observation (Olkowski et al. 1992).

It is a reasonable assumption that animals fed excess S may have a greater demand for thiamine due to a toxic effect of excess dietary S. The increased metabolic demand for thiamine was postulated by Loew (1975) as a possible etiological factor of PEM. Hence, is what is considered "normal" indeed adequate under the circumstances of an increased requirement? If the hypothesis of increased demand for thiamine in treated and/or affected animals is accepted, then the assumption of thiamine adequacy on the basis of comparative (i.e. normal or not-treated vs affected or treated) measurements of thiamine concentration and the activities of thiamine dependent enzymes may be invalid. Reasonably, the "normal" thiamine status established for animals not exposed to excess S, in the situation of increased demand for thiamine in the S treated animals, should be considered as indicative of deficiency. Evidence supporting a higher requirement for thiamine in animals exposed to high dietary S was previously reported (Olkowski et al. 1991).

#### **2.1.4.4 Hypothesis for S toxicity**

In ruminants, sulfides and sulfites are produced in large quantities as natural metabolites of S and there is a potential for synthesis of S-derived free radicals,

both in the rumen and systemically. Interestingly, the toxic effects in this species have unique features not seen in other species. The brain is the target organ. Perhaps the pathogenesis of S toxicity attests that ruminants likely have some unique features of S metabolism resulting in such specific lesions.

Potentially the toxicity of S or S metabolites may be an important factor in the development of brain lesions. Brain tissue, because of its high lipid content, would be vulnerable to damage by free radicals. It is conceivable that, in S toxicity, the actual structural damage may be an effect of oxidative damage to vital cellular structures inflicted by S metabolites such as sulfides, sulfites and/or their more reactive derivatives. Furthermore, considering that the neural tissue has substantially less damage repairing and regenerating ability than other tissues, it is possible that even a mild thiamine insufficiency, under conditions of increased requirement, may lead to impairment of the detoxifying processes and could result in brain damage. Sulfite derived radicals can be formed through a catalytic reaction of peroxidases and are postulated to cause lipid peroxidation and damage to biological membranes (Mottley and Mason 1988). A deficit of thiamine can increase the intensity of lipid peroxidation (Lychko et al. 1987). In a more recent study, Ozawa and Hanaki (1990) suggested the formation of thiosulfate and sulfide radical anions from the reactions of hydrogen peroxide with thiosulphate and sulfide ions respectively. The involvement of S containing free radical anions may be important in the toxicity of S to the living cells.

Undoubtedly, the problem is more complex and the lesions caused by toxic effects of S are likely due to a combination of several factors. One highly probable factor may be the problem of a local deficit of thiamine due to more intensive metabolic demand for thiamine which can be further hampered by interference with thiamine bioavailability.

#### **2.1.4.5 Thiamine status and bioavailability**

In clinical cases (Gooneratne et al. 1989a, Gould et al. 1991, Olkowski and Christensen 1992), it appears that there is tendency to increase the amount of circulating thiamine. Interestingly, blood thiamine concentrations in sheep fed the high S diet were in fact slightly higher than in sheep fed the low S diet. A similar trend was found in the study of Goetsch and Owens (1987). Also in our study (Gooneratne et al. 1989b, Olkowski and Christensen 1992) animals which developed clinical signs of PEM tended to have higher thiamine concentrations in their blood than apparently normal animals. The data presented by Gould et al. (1991) indicates that this apparent paradox is not peculiar to the present study.

It is not clear what causes the elevation of blood thiamine, although the possibility of interference by some form of antimetabolite seems plausible. The *in vitro* study showed that in the S treated rumen content cultures there was considerably less thiamine detected by a biological method as compared to comparison to HPLC method. Hence, there was a factor which influenced the bioavailability of thiamine. Hypothetically, if such a factor is indeed produced in the rumen and absorbed *in vivo*, and if this factor obstructs the bioavailability

of thiamine in tissues, then metabolic starvation for thiamine could produce a feedback-homeostatic mechanism which possibly could: a) intensify absorption of thiamine; b) decrease excretion; c) release thiamine from storage or less demanding tissues to the circulation to provide better supply to vital organs. The effect of this would be an increase in blood thiamine concentration. The above described mechanisms (well documented in nutritional physiology) are natural reactions of the body in similar nutritional situations.

Alternatively, excess sulfide produced in the rumen could form complexes with thiamine (likely in its thiazole open ring) in a way that "fools" the bacteria, leading to increased bacterial synthesis. The highly reducing environment of the rumen would be probably quite favourable for formation of "S-thiamine complexes", however, these complexes could dissociate in the small intestines providing more thiamine for absorption.

The above hypotheses are supported by the results obtained from the in vitro study. Excess S does not appear to affect ruminal thiamine synthesis, however, there is also an apparent decreased bioavailability of thiamine after prolonged incubation in the presence of S (see Chapter III).

#### **2.1.4.5.1. Thiamine antimetabolite**

The concept of formation of a thiamine antimetabolite in the rumen as a potential cause of PEM was introduced previously (Edwin and Jackman 1982) and is particularly interesting in the context of the S induced cases of PEM. It has been shown in vitro that the sulfite ion catalyses a reaction where the elimination

of the thiazole moiety from the thiamine molecule generates a reactive electrophile that is then captured by a nucleophile to give rise to a substitution product (Zoltewicz et al. 1982). A number of naturally occurring compounds (Brent and Bartley 1984) can serve as co-substrates for this reaction. Hence there is a possibility that the sulfite ion can act as a catalyst in the formation of thiamine-like (analogue) compounds in the rumen.

Thiamine analogues can act as antimetabolites interfering with the metabolism of thiamine. Aspects of thiamine metabolism such as intestinal transport (Casirola et al. 1988), brain uptake (Nose et al. 1974) and inhibition of thiamine dependent enzymes (Gibson et al. 1984, Butterworth et al. 1985,1986, Guiguere and Butterworth 1987) are known to be affected by pyriothiamine. Thiamine inadequacy is observed in animals treated with amprolium (Loew and Dunlop 1972a, Cymbaluk et al. 1978). Thus, if thiamine-like compounds are indeed produced in the rumen of animals exposed to excess S, one can expect interference with thiamine metabolism. Our in vitro study (Chapter III) showed that the bioavailability of thiamine is affected in S treated rumen content cultures. It would be interesting to define what causes this problem.

#### **2.1.4.5.2 Possibility of antimetabolite in the present study**

This study indicates that S induced PEM is not likely caused by an overt thiamine deficiency based on the criteria commonly used to assess thiamine status. However, some evidence to support the hypothesis of thiamine antimetabolite involvement was found. Notably, despite higher blood thiamine

concentrations in sheep fed the high S diet, the erythrocyte TPP effect increased indicating a metabolic thiamine insufficiency. Similar findings have previously been reported in horses upon administration of the thiamine analogue amprolium (Cymbaluk et al. 1978). We found higher blood thiamine concentrations in sheep fed high S diets unsupplemented with thiamine compared to these fed a low S diet. Increased blood thiamine was also reported with the administration of amprolium in calves (Lilja 1973) and sheep (Loew and Dunlop 1972a).

A rather intriguing observation in the present study was the apparent poor correlation among blood thiamine concentrations, TK activities, and TPP effects (Olkowski and Christensen 1992, see Chapter I). A number of animals had relatively high blood thiamine concentrations and high TPP effect values. In several instances low TK activity occurred concurrently with normal blood thiamine concentration, and the addition of TPP did not result in an increase of TK activity. Hypothetically, the above could occur in the scenario if there was: (i) a lack of synthesis of apoenzyme, or (ii) inadequate phosphorylation of thiamine to TPP, or (iii) the coenzyme (TPP) had reduced activity, or (iv) access to the active center of the apoenzyme was blocked/occupied, or (v) coenzyme (TPP) could not be fitted into the active center of the apoenzyme. Although point (i) cannot be disproved, the detailed analysis of the status of thiamine phosphates provided by Gould et al. (1991) tends to dismiss point (ii). However, points (iii), (iv), and (v) elegantly fit the possibility of interference with bioavailability.

Reasonably, metabolic thiamine deficiency paralleled with relatively high blood thiamine levels may result only from hindered bioavailability of the vitamin.

It has to be stressed that ruminal synthesis of thiamine antimetabolites in amounts which are required to cause severe effects as observed in experiments with amprolium (Loew and Dunlop 1972a) or pyrithiamine (Gibson et al. 1984, Butterworth et al. 1985,1986, Guiguere and Butterworth 1987) is highly improbable.

#### **2.1.4.6 Is higher blood and tissue thiamine concentration required?**

Perhaps the assumption of measuring thiamine adequacy on the basis of thiamine concentration and the activities of thiamine dependent enzymes is not applicable to every situation. There is a possibility that a higher concentration of thiamine may be required in order to prevent toxic effects. Armett and Cooper (1965) found that treatment of the vagus nerve with pyrithiamine increased the amplitude of the compound action potential and obliterated the post tetanic hyperpolarization. However, when the vagus nerve was pre-incubated in solution containing thiamine, the treatment with pyrithiamine was without effect. Similarly, pyrithiamine displaced thiamine from the original nerve preparation, but not from a thiamine pre-treated preparation (Cooper 1968). Thus, the action of pyrithiamine could be directly related to thiamine in these experiments. Further, free thiamine is thought to protect the cell by scavenging potentially toxic intermediates generated by the myeloperoxidase/H<sub>2</sub>O<sub>2</sub>/halide system (Theron et al. 1981). Hence, the concentration of thiamine beyond the level necessary to

maintain the activity of thiamine dependent enzymes may be required in the face of a toxic insult. Indeed, this study and others (cited above) indicate that the organism of affected animals tends to increase concentration of circulating thiamine.

The above may be of particular interest in view of our results. It appears that the protective effect of thiamine can be related to its concentration in the brain tissue. Higher concentrations of free thiamine may be required to endure the insult of S metabolites. In view of the recent findings (Sager et al. 1990, Gould et al. 1991, Olkowski et al. 1992) it appears that the etiology of PEM based on the concept of overt thiamine deficiency may require revision. The metabolic functions of thiamine other than its role as a TPP co-enzyme deserve more consideration in the pathogenesis of PEM. The possible involvement of thiamine in regulating ion uptake (Bettendorff et al. 1990),  $\text{Na}^+, \text{K}^+$  ATPase activity (Matsuda and Iwata 1987), and water movement across the cellular membrane (Brown 1982) may be important in the pathogenesis of PEM, and provide some basis to explain the therapeutic effects of thiamine.

Thus, inadequate thiamine concentration in the brain may be of importance for the development of brain edema, and the manifestation of clinical signs may depend on the presence and/or severity of brain edema. It is notable that thiamine supplementation in sheep alleviated electrophysiological alterations and prevented manifestation of clinical signs, although it did not completely prevent morphological changes. There is no doubt here that higher thiamine status had

preventative effect. It is not known if further increases in thiamine status would completely protect against S toxicity.

## **2.2 Hydrogen sulfide involvement in S induced PEM**

The possibility that the toxic effects of S in ruminants may be associated with inhalation of hydrogen sulfide gas produced in the rumen and inhaled during eructation has been debated. This subject was discussed in detail in the literature review. Such toxicity appears possible when the S is infused in very large quantities and in a specific form. However, is this kind of toxicity possible at lower level chronic dietary exposure? Recently, Gould et al. (1991) put forward the hypothesis that eructated and inhaled hydrogen sulfide gas may be implicated in the development of brain lesions in animals exposed to excess dietary S. Inasmuch as the neurotoxicity of hydrogen sulfide is unquestioned, the concentration of hydrogen sulfide in the eructated gas may not be high enough to be harmful. Likely, the gas is accumulated in the rumen in insufficient concentration to exert a toxic effect per se. Further, since the respiratory motion is consistently altered at the onset of eructation, with a momentary cessation of activity being the rule, and eructation occurs in conjunction with rumination at which time due to chewing motions the mouth is poorly sealed (Dougherty et al. 1965), the probability of inhalation of toxic levels appears to be quite remote indeed. No doubt, some of the eructated hydrogen sulfide gas is inhaled, but to what extent it contributes to the pathogenesis of S induced syndrome remains to be seen.

### **3.1 Practical aspects of nutritional S toxicity**

#### **3.1.1 Are we facing an epidemic of S related problems?**

The findings on S toxicity in past research are not consistent with findings during the last decade. Notably, some authors fed S levels several fold higher than those used in the present study with no observable deleterious effects (Chalupa et al. 1971, Embry et al. 1959), or observed reduced performance (Weeth and Hunter 1971, Weeth and Capps 1972, Bouchard and Conrad 1973). Raisbeck (1982) investigated epidemiology of S induced PEM in cattle. His study concluded that cattle fed excess S were 43 times more likely to develop PEM than those fed normal S diets. A number of recent reports have shown that PEM can be induced upon feeding excess dietary S (Sadler et al. 1983; Rousseaux et al. 1991, Gould et al. 1991). Polioencephalomalacia was also incidently observed during feeding trials where excess dietary S was fed to cattle (Khan et al. 1987; Gibson et al. 1988). Further, evidence from veterinary practice (Hibbs and Thilsted 1983, Harries 1987, Gooneratne et al. 1989a) attests that there is an increasing trend to recognize the problem of S toxicity. It appears that the decreased tolerance of cattle for S and the drastic change in the clinical picture is a recent phenomenon, since the recognition of the problem is not documented in older literature.

#### **3.1.2 Potential Impact on the Livestock Industry**

There is no data available to asses the prevalence of S-associated PEM. During the course of our study (1987-1991) in the province of Saskatchewan, on

the basis of personal communication and reports (very limited in number) we have documented three instances of major outbreaks of PEM associated with high sulfate water. In each case there were losses of several animals. The recognition of marginal thiamine deficiency associated with excess dietary S, and general metabolic disorders associated with thiamine deficiency may not easily be diagnosed.

The potential economic impact may be difficult to assess. The severity of the problem of nutritional S toxicity may depend on numerous nutritional and management factors. Further, because of the complexity of nutritional interactions it would be very difficult to calculate monetary losses. Some authors noted a decreased daily weight gain (DG) by about 10 - 20 % in animals fed excess S. In the study of Rumsey (1978) steers fed a ration with 0.42% S had DG some 20% lower than those fed 0.14% S ration, and animals fed 0.98% S ration were losing weight at a rate 0.04 kg daily. In growing billy goats, bulls and heifers, the supplementation of 10 g elemental S/kg ration DM reduced the DG by about 15% (Anke et al. 1989). Subclinical problems associated with excess dietary S do occur, however, there is no data available about their significance. Subclinical toxicities, likely being associated with secondary nutritional deficiencies, may represent a plethora of metabolic disorders.

In view of the abundance of high sulfate water available to livestock in Saskatchewan the problems of a S-induced nervous disorder and S-associated thiamine deficiency may be of economic importance. A thorough epidemiological study of the problem is highly recommended.

## CONCLUSIONS

It can be concluded from these studies that excess S causes a number of metabolic disturbances in ruminants. Several of them were not described previously and are yet to be verified by others.

- 1) The evidence from the brain evoked potentials experiment indicates alterations of electrophysiological events and that a thiamine deficit is one of the key factors associated with abnormal BAER recordings. Further study is required to examine the metabolic pathogenesis of these abnormal neurophysiological findings.
- 2) Changes in several biochemical parameters observed in high S-related PEM in sheep and cattle appear to be similar to those observed in naturally occurring and amprolium-induced PEM in ruminants. No pathognomonic diagnostic markers enabling early recognition of the problem were found.
- 3) The present study has shown that interactions among S, Mo, Cu and thiamine occur in the GI tract and systemically. Copper status was affected by excess Mo in the high S diet. Copper status likely plays an important role in the metabolism of thiamine. Copper deficiency in ruminants could predispose an animal to thiamine deficiency. Excess dietary Mo may exacerbate the effects of high S on Cu and thiamine status. Further studies are warranted in order to elucidate the mechanism by which these nutrients interact.

- 4) High concentrations of sulfate in the drinking water are associated with low blood thiamine concentrations in beef cattle. A sub-population of beef cattle in Saskatchewan is predisposed to marginal deficiency or deficiency of thiamine. Such a deficiency state is most likely to occur in areas where animals are exposed to high sulfate water. Considering the prevalence of high sulfate water on the Canadian Prairies, routine thiamine supplementation to exposed livestock is recommended.
- 5) Thiamine status indicative of deficiency is not necessarily the cause of PEM, although thiamine inadequacy may increase the risk of developing the clinical signs of PEM. However, enhanced thiamine status prevents development of clinical signs of PEM in animals fed excess S.
- 6) This study indicates that S induced PEM is not likely caused by overt thiamine deficiency based on commonly used criteria. The commonly used indices may not adequately reflect the requirement for thiamine. The morphological damage is not likely due to metabolic effects of decreased activities of TPP-dependent enzymes. Thiamine more likely plays some protective role apart from its metabolic effect through thiamine dependent enzymes. It is possible that in the situation of increased demand for thiamine, even a mild thiamine deficiency can lead to impairment in detoxifying processes and resultant brain damage. The involvement of the S metabolites; sulfite, sulfide, and/or their free radicals in the morphological damage to the brain tissue is highly probable.

- 7) Evidence was obtained that S affects the bioavailability of thiamine in the rumen. Synthesis of thiamine antimetabolites in the rumen is probable. Some indirect evidence of possible systemic effects from a factor interfering with thiamine metabolism was observed in the present study. The benefits of thiamine supplementation to animals exposed to excess dietary S were obvious.

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## APPENDIX

Microbiological methods of thiamine measurement in biological material. A.A. Olkowski and S.R. Gooneratne. *Int. J. Vit. Nutr. Res.* 1992. (In press).

## MICROBIOLOGICAL METHODS OF THIAMINE MEASUREMENT IN BIOLOGICAL MATERIAL

Olkowski, A.A. and Gooneratne, S.R. 1992. Microbiological methods of thiamine measurement in biological material. *Int. J. Vit. Nutr. Res.* (In press).

### SUMMARY

Semiautomated, microbiological methods for the determination of thiamine in a variety of biological material are presented. Thiamine content was measured in bovine, ovine and rat blood, plasma, bile, urine, duodenal fluid, liver, kidney and brain. Thiamine was extracted from samples by mild acid digestion (transaconitate pH 4.0) at 115°C for 20 min. Microbiologic assays utilizing *Lactobacillus fermenti* and *Ochromonas danica* were performed on 96-well microplates and growth, as monitored by optical density, was directly read on a microplate reader at 650 nm. Particular attention was given to the validation of the two methods in terms of accuracy, reproducibility and recovery. The microbiological methods described here are specific, sensitive, and ensure good reproducibility. The methods can be used to analyse a large number of samples rapidly and at low cost.

## INTRODUCTION

Several methods for the measurement of thiamine in biological material have been described. The thiochrome assay, one of the oldest thiamine assay systems (Hennessy and Cerecedo 1939, Burch et al 1952), depends on the oxidation of thiamine by ferricyanide into thiochrome which is measured fluorometrically. The main drawbacks of this method include differences in fluorescence of thiamine metabolites (Penttinen 1976) and interference by non-thiamine natural fluorophores similar to thiochrome (Loew et al 1972), which are often present in biological material (Loew & Dunlop 1972). Hence, the use of the thiochrome method in measurement of thiamine concentration in biological material is prone to error and hence questionable.

High performance liquid chromatography (HPLC) techniques for thiamine determination (Rindi and Giuseppe 1961, Ishi et al 1979, Bötticher and Bötticher 1986, Finglas and Faulks 1987) have been gaining popularity in recent years. These methods are sensitive, but there are numerous technical difficulties encountered in this procedure. The detection in HPLC methods is based on thiochrome, and unless a sophisticated purification system is employed, the analytical criteria do not ensure that the eluted fluorophores are exclusively thiamine derived compounds (Matsuda and Cooper 1981, Itawa et al 1985). Biological assays using the phytoflagellate *Ochromonas danica* (Baker and Frank 1968) and the bacterium, *Lactobacillus fermenti* (Sarett and Cheldelin 1944), are also commonly used procedures for estimation of thiamine. However, the usefulness

and worth of the biological methods may be not fully appreciated by researchers. The phytoflagellate and bacterial methods described here are modifications of the assays of Baker and Frank (1968) and Sarett and Cheldelin (1944), respectively.

## **MATERIAL AND METHODS**

### **Materials and procedures common for both methods**

**Trans-aconitate buffer:** Trans-aconitic acid (Sigma) 5g, water 1000ml, (adjusted to pH 4.0 with KOH).

**Thiamine stock solution:** Thiamine hydrochloride (thiamine HCl) 1 mg/L in trans-aconitate buffer.

**Preparation of standards:** Standards containing 0.75, 1.5, 3.0, 6.0 and 12.0  $\mu\text{g}$  thiamine HCl/L were prepared by diluting the thiamine stock solution in aconitate buffer and autoclaving at 115°C for 20 min.

**Preparation of samples:** Samples of blood, plasma, bile, urine and tissues were stored at -20°C. Tissues were homogenized (Brinkmann PT 10/35 homogenizer, Brinkman Instruments, Rexdale, Ontario) in the aconitate buffer at a ratio 1:10 w/v. Samples were added to aconitate buffer in glass tubes at a ratio of aconitate buffer to blood, plasma or bile at 4:1 v/v, to urine at 99:1 v/v, and to tissues homogenates at 4:1 v/v. All tubes were then vortexed, capped and autoclaved at 115°C for 20 min. The extracts were then vortexed while still hot and centrifuged at 2000g for 15 min to obtain a clear supernatant containing thiamine. This thiamine extract was used for the assays. Some supernatants had to be further diluted in order to be within the linearity limits of the standard curve.

Such supernatants were autoclaved again (after appropriate dilution) at 110°C for 1 min. From this step onwards, all equipment and materials used in the assay were sterilized and all procedures were carried out using sterile techniques.

**Reference sample:** To establish a reference value, 1 L of sheep plasma was stored as 1 mL allotments at -20°C. Initially, 25 assays were carried out (in quadruplicates) and the mean was established as an internal reference value. This reference plasma standard was used with each assay to determine inter and intra assays coefficients of variations (all less than 8%).

**Ochromonas danica method.** A stock culture of *Ochromonas danica* [American Type Tissue Collection (ATCC No.30004) ATCC laboratory Rockville, MA] was maintained on thioglycolate medium BBL-11260 (Microbiology Systems, Becton Dickinson, Cockeysville, MA) according to ATCC instruction. Three days prior to the assay, the stock culture of *O.danica* was subcultured on BBL-11260 medium and transferred to an incubator illuminated with two 15-watt fluorescent lamps and a 60-watt incandescent lamp, with temperature maintained at 25°C. A basic assay medium was prepared according to the method of Baker and Frank (1968) and the final assay medium was prepared by diluting the basic assay medium in distilled water at a ratio of 1.25:1. The *O. danica* inoculum was prepared by adding a few drops of 3 d old subculture to 5 ml of isotonic saline followed by vortexing and centrifugation at 1000g for 5 min. The supernatant was discarded and the pellet containing the protozoa was then washed twice in saline followed by resuspension in saline (approximately 2 ml) at a concentration equivalent to

1.0 optical density (OD) unit at 650 nm wavelength. The final assay solution was prepared by mixing four drops of the protozoal inoculum with 20 mL of final assay medium. Aliquots (160  $\mu$ l) of this final assay solution were then added to each of the 96 wells in the microplate (Falcon 3792, Becton Dickinson Labware, Oxnard, CA) and allowed to remain at room temperature for 30 min to condition the microplate prior to sample addition. Equal volumes (40  $\mu$ l) of blank (aconitate buffer), standards, reference sample and unknown samples were then added to the wells in the microplate in quadruplicates, followed by incubation at 30°C with illumination for 96 h. At the end of the incubation period, microplates were vortexed and read at 650 nm on the microplate reader (V max, Molecular Devices Corporation, Palo Alto, CA). All calculations were carried out using a software program, 'SoftMax' (Molecular Devices Corporation, Palo Alto, CA), interfaced to the microplate reader.

**Lactobacillus fermenti method.** Long term cultures of *Lactobacillus fermenti* (ATCC No. 9338, Difco Laboratories, Detroit, MI) were maintained as stab cultures on Lactobacilli agar (Difco Lab, Detroit, MI) at 4°C. Stock cultures were prepared by transferring the stab culture to a Lactobacilli broth (Difco Laboratories, Detroit, Michigan), and incubating at 37°C for 18 - 20 h. These stock cultures could be stored at 4°C for up to 2 weeks. Stock cultures were maintained by transfer to a Lactobacilli broth fortnightly. One day prior to the assay, a stock culture of *L. fermenti* was transferred to Lactobacilli broth and incubated at 37°C for 16-18 hours. The bacterial inoculum was prepared by centrifuging the broth

containing the culture at 2000g for 5 min. The supernatant was discarded and the remaining pellet, containing bacteria, was washed three times in isotonic saline and resuspended in saline at a concentration equivalent to 1.0 OD unit at 650 nm. The final assay medium was prepared by mixing 8.5 g of dehydrated Lacto thiamin assay medium (Difco Lab, Detroit, MI) with 160 ml of distilled water and boiling for 3 min. This was distributed into 20 ml allotments and sterilized at 110°C for 1 min. The final assay solution was prepared by mixing four drops of bacterial inoculum with 20 ml of final assay medium. Aliquots (160 µl) of this solution were then added to each of the 96 wells in the microplate 30 min prior to adding the samples. Equal volumes (40 µl) of blank (aconitate buffer), standards, reference sample and unknown samples were then added into the wells of the microplate in quadruplicates, followed by incubation at 37°C for 16-18 h. Following this, the microplates were vortexed and read directly on the microplate reader as described above.

## RESULTS

Since there is no unanimity in the literature as to units of expression of thiamine concentration in samples, all thiamine values presented in this manuscript are expressed in terms of thiamine HCl. To convert these values to a concentration of thiamine, a conversion factor of 0.7868 needs to be used to express on a weight basis and a conversion factor of 2.9649 to express on molar basis. Typical standard curves for *L. fermenti* and *O. danica* methods are shown in Figs. 1 and 2, respectively. With both methods, linearity of the standard curve

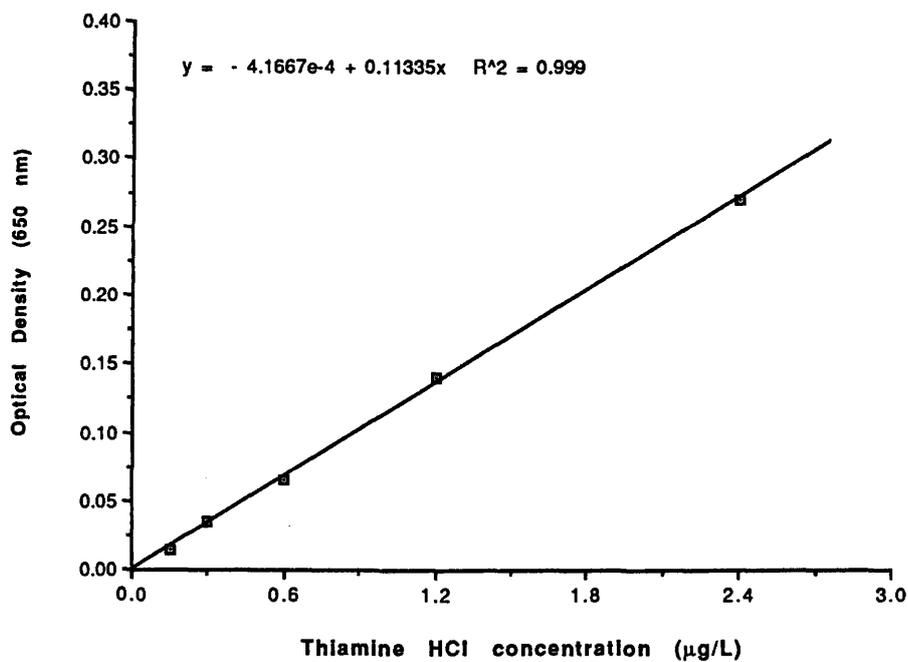


Fig. 1. A typical standard curve for estimation of thiamine with *Lactobacillus fermenti* method.

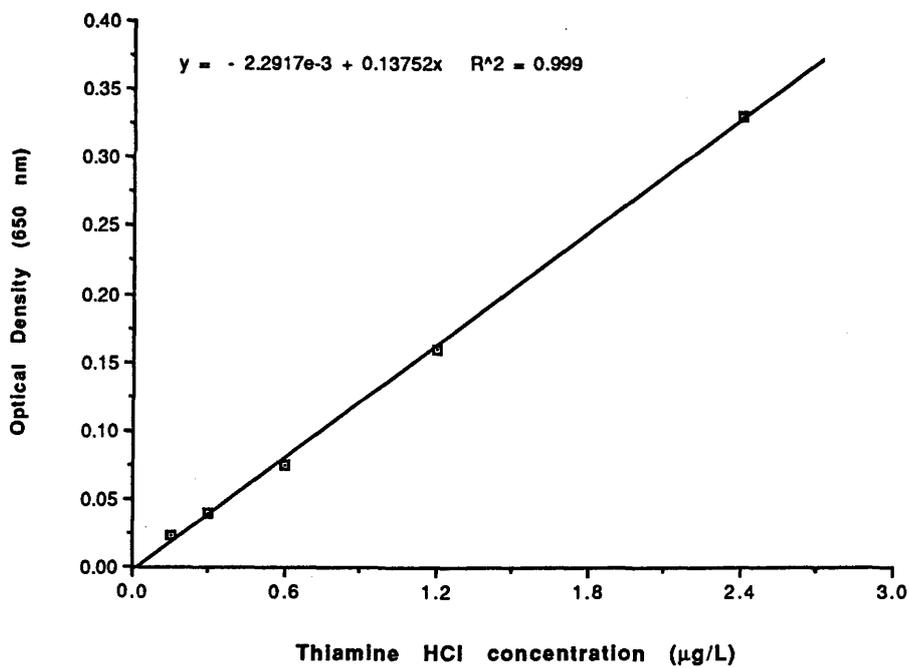


Fig. 2. A typical standard curve for estimation of thiamine with *Ochromonas danica* method.

was maintained up to 4.8  $\mu\text{g}$  thiamine HCl/L, which is equivalent to 24  $\mu\text{g}$  thiamine HCl/L in the plated sample since the dilution in the microplate was 5 fold. The lowest detection limit (effective in sample) for both methods was 0.75  $\mu\text{g}$  thiamine HCl/L. The correlation coefficient of the standard curve obtained from either assay was routinely more than 0.999.

Table I shows a comparison of the two methods. The result of both *O. danica* and *L. fermenti* methods are comparable for blood, brain, and plasma of sheep. Recovery assays, carried out by adding known amounts of thiamine HCl to blood, plasma, urine, and liver homogenate of sheep, using the *L. fermenti* method are shown in Table II. Recoveries ranged from 93 to 110%. Similar results were obtained for the *O. danica* method. Assays performed with *L. fermenti* on microplates were compared to the more traditional method using glass tubes (Table III). Both the amounts and coefficients of variations were similar. Table IV shows thiamine concentration in a wide variety of biological samples from cattle, sheep and rat.

## DISCUSSION

The common disadvantages most often emphasized in the literature concerning microbiologic methods are poor reproducibility and lack of automation (Bötticher and Bötticher, 1986). A number of factors can affect reproducibility of a microbiological assay, namely improper technique, use of biologically impure strains for assays, and contaminated or non defined media.

TABLE I. Comparison of the two microbiological methods (*O. danica* and *L. fermenti*) in measurement of thiamine content in sheep blood, plasma ( $\mu\text{g/L}$ ), and brain ( $\text{mg/kg}$ ).

Sample *	<i>L. fermenti</i>		<i>O. danica</i>	
	Mean	CV <sup>†</sup>	Mean	CV
Blood 1)	111.95	2.2	103.50	2.3
2)	114.65	3.1	109.90	4.5
3)	87.70	4.7	86.80	3.7
Brain 1)	1.20	5.6	1.29	2.5
2)	0.98	6.8	0.97	7.1
3)	1.04	4.3	1.06	4.7
Plasma (reference)	21.80	5.8	21.65	6.8

\* 3 samples each of blood, brain and plasma reference were assayed in quadruplicates.

<sup>†</sup> CV refers to intra assay coefficient of variation.

TABLE II. Results of thiamine recovery tests using *L. fermenti* method.

Sample	Thiamine ( $\mu\text{g/L}$ )				CV <sup>†</sup>	% Recovery
	Original Concentration	Amount Added	Final Concentration			
Plasma	21.35	20.0	43.35	4.8	110	
	21.35	40.0	63.75	3.8	106	
	21.35	60.0	79.95	3.1	98	
Blood	59.88	20.0	80.88	5.1	105	
	59.88	40.0	97.28	4.6	93	
	59.88	60.0	122.08	2.1	104	
Urine	1935.00	1000.0	2987.00	4.1	105	
	1935.00	1500.0	3486.00	4.5	103	
	1935.00	2000.0	4008.00	3.2	104	
Liver homogenate	1457.00	300.0	1743.00	6.8	95	
	1457.00	400.0	1864.00	5.3	102	
	1457.00	500.0	1953.00	2.4	99	

Known amount of thiamine hydrochloride was added to either plasma, blood, urine or liver homogenate and the % of recovered thiamine calculated using the *L. fermenti* assay. % recovery was calculated using formulae:  $(\text{final conc.} - \text{original conc}) \times 100 / \text{amount added}$ .

<sup>†</sup> CV refers to intra assay coefficient of variation.

TABLE III. Comparison of *L. fermenti* assay performed in glass tubes and in microplates.

Blood Sample No.	Glass Tube		Microplate	
	( $\mu\text{g/L}$ )	CV <sup>†</sup>	( $\mu\text{g/L}$ )	CV
1	76.18	2.4	75.72	2.7
2	29.57	6.3	29.04	4.6
3	46.34	4.5	46.20	3.7
4	37.46	3.5	36.12	4.5

<sup>†</sup> CV refers to intra assay coefficient of variation.

TABLE IV. Thiamine concentration in normal biological material from cattle, sheep and rats as assayed by the *L. fermenti* method.

Sample and species	Thiamine concentration *		
	mean	SD	range
<b>Whole blood</b> ( $\mu\text{g/L}$ )			
- cattle	78.80	22.32	50 - 112
- sheep	85.20	12.98	68 - 103
<b>Plasma</b> ( $\mu\text{g/L}$ )			
- rat	268.80	37.25	212 - 320
<b>Urine</b> ( $\mu\text{g/L}$ )			
- sheep	2764.20	1825.99	569 - 4775
- cattle	194.20	94.47	101 - 291
<b>Bile</b> ( $\mu\text{g/L}$ )			
- sheep	42.46	16.18	21.50 - 67.50
- cattle	11.20	4.07	5.60 - 15.20
<b>Duodenal fluid</b> (mg/L)			
- sheep	1.15	0.54	0.70 - 2.50
<b>Sheep tissues</b> (mg/kg)			
- liver	7.50	1.68	5.40 - 9.30
- kidney (cortex)	4.10	0.75	3.20 - 5.50
- brain (grey matter)	1.10	0.15	0.96 - 1.37
- heart	4.60	0.76	3.60 - 5.50
<b>Rat tissues</b> (mg/kg)			
- liver	9.10	4.06	5.40 - 15.90
- brain (whole)	4.40	1.36	2.80 - 6.60

\* Refers to mean, standard deviation (SD), and range for 6 samples for each category of each species.

Experiments described here indicate that both *Lactobacillus* and *Ochromonas* methods, if carried out properly, will demonstrate good reproducibility, specificity and sensitivity. The use of a 96-well microplate for routine microbiological thiamine assays allows processing of a large number of samples rapidly, efficiently and at a low cost.

The measurements of thiamine using microbiological methods are as sensitive as the HPLC based systems. The values for tissue (liver, brain, kidney, heart) thiamine concentration obtained using HPLC methods (Rindi and Giuseppe 1961, Ishii et al 1979, Iwata et al 1988) are in accord with our measurements.

The analyses done in our laboratory show a high degree of agreement between the protozoal and bacterial methods for thiamine estimation in tissues. However, in urine, bile, rumen and duodenal content, the bacterial method routinely detected more (wide range of variation) thiamine than the protozoal method. The data from several thousand blood samples from cattle and sheep indicated that the bacterial method consistently detects 5-10%, and less frequently up to 25%, more thiamine in the blood than the protozoal method.

In our data base we have several documented cases where in blood from cattle and sheep suspected of thiamine deficiency, the protozoal assay detected 2 to 5 times less thiamine than the bacterial assay (Olkowski and Gooneratne, unpublished observation), some examples being shown in Fig. 3.

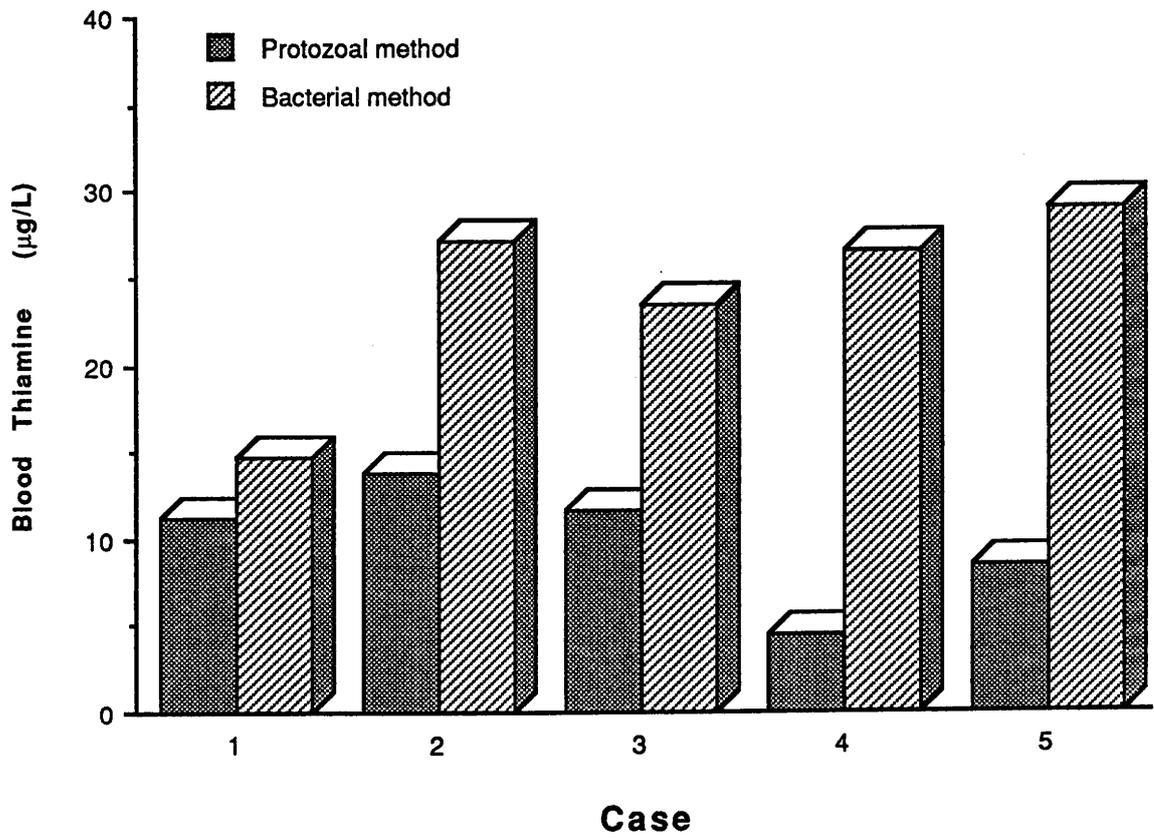


Fig. 3. Differences in blood thiamine concentration as detected by protozoal and bacterial methods in cattle and sheep suspected of thiamine deficiency.

Two heifers (case 2 and 3) and two sheep (case 4 and 5) developed classical clinical signs of thiamine deficiency. The reason for such a discrepancy between the bacterial and protozoal methods in detecting thiamine in blood, urine, bile and digestive content is not clear but we presume that bacteria may be able to recognize a variety of thiamine metabolites, or be less affected by potential factors obstructing bio-availability of thiamine. In contrast, *O.danica* having a mammalian-like requirement for thiamine, is capable of utilizing only the intact and biologically available thiamine (Baker and Frank 1968).

It is notable that in these assays the growth inhibition of *O. danica* could be overcome by addition of an excess of thiamineHCl to the sample. Similar effects were observed when aqueous thiamine inactivated by prolonged autoclaving was added to blood samples. This provides evidence that biologically available thiamine was the limiting factor.

Proper assessment of thiamine status may be very difficult and in this context the bio-availability deserves special consideration. Thiamine is unique in its biological specificity and even very minor alterations to the thiamine molecule can render it biologically inactive or even produce powerful anti-metabolites (Rogers 1962). At least 22 different metabolites of thiamine have been found in rat and rabbit urine (Neal and Pearson 1964, Pearson et al 1966). Thiamine metabolites and/or analogues may interfere with intestinal transport of thiamine (Casirola et al 1988), brain uptake (Nose et al 1974) and inhibit thiamine dependent enzymes (Gibson et al 1984). In addition, there is a plethora of naturally occurring

compounds with anti-thiamine activity (Hilker and Somogyi 1982). In ruminants, thiamine analogues may be produced in the rumen by the catalytic reaction of the enzyme thiaminase I (Edwin and Jackman 1982).

The problem of bioavailability in assessment of thiamine status cannot be ignored, particularly in *in vivo* situations. HPLC methods can accurately measure the amount of thiamine, but this provides rather limited information since the presence of thiamine metabolites, biologically inactive thiamine or other substances potentially affecting bioavailability of thiamine is not taken into consideration. The presence of thiamine in a biological sample at a level recognized as adequate does not necessarily mean that the vitamin is completely available to the organism. Furthermore, factors influencing bioavailability may be difficult to control or predict. Therefore, evaluation of thiamine status for nutritional or diagnostic purposes using microbiological methods has definite advantages. The biological methods may provide good assessment of bioavailability.

In conclusion, the two microbiological methods of thiamine measurement presented here are simple, can be semi-automated, have a low detection limit and a high degree of precision and reproducibility. The microbiological methods are appropriate for thiamine measurement for nutritional and clinical purposes. Either assay (*L. fermenti* or *O. danica*) could be used to detect a thiamine deficiency state in a tissue. However, the protozoal method appeared a better indicator of biologically active thiamine in specimens likely to contain thiamine

metabolites or other compounds having anti-thiamine activity. Those would include blood, urine, bile, intestinal content, rumen content or food. Both microorganisms require thiamine for growth, but considering the apparent ability of bacteria to be less affected by "anti-thiamine activity" this feature can be used to assess bioavailability of thiamine. Thus, when both assays are performed on the same samples, a comparison of the results from the bacterial and protozoal methods would enable one to distinguish whether thiamine deficiency was caused by a primary deficiency of thiamine per se, or was related to the bioavailability of thiamine.

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