EVALUATION OF SULFUR AND THIAMINE METABOLISM, SHORT CHAIN FATTY ACID ABSORPTION, AND MINERAL STATUS IN BEEF CATTLE FED HIGH DIETARY SULFUR

A Thesis Submitted to the

College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Large Animal Clinical Sciences

Western College of Veterinary Medicine

University of Saskatchewan

Saskatoon, SK

By

Samat Amat

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of Department of Large Animal Clinical Sciences

Western College of Veterinary Medicine

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5B4

ABSTRACT

A small pen study, a metabolic trial and a field observation study were conducted to evaluate sulfur (S) and thiamine metabolism, short chain fatty acid (SCFA) absorption, and mineral status in beef cattle fed high dietary S. In the small pen study, the effect of feeding corn (CDDGS), wheat (WDDGS) or a 50:50 corn:wheat blend (BDDGS) dried distillers grains with solubles (DDGS) on serum sulfate level of feedlot steers was evaluated using 288 crossbred steers (273.9 ± 18.5kg) in a completely randomized design. The steers were backgrounded and finished. The control backgrounding diet consisted of 34.3% barley grain, 26.0% brome grass hay, 10.3% barley straw, 22.8% barley silage, and 6.7% supplement (DM). For the three treatment diets, 17% of the barley grain was replaced with DDGS. Sulfur concentrations of control, BDDGS, CDDGS and WDDGS diets in the backgrounding phase were 0.2, 0.23, 0.31 and 0.33% (DM), respectively. The control finishing diet was 86.8% barley grain, 5.8% supplement and 7.4% barley silage (DM), and the three DDGS treatments included replacement of 40% of barley grain with an equal amount of DDGS. The corresponding sulfur concentrations for control, BDDGS, CDDGS and WDDGS diets in were 0.2, 0.33, 0.51 and 0.65% (DM), respectively. Corn DDGS or WDDGS cattle exhibited higher ($P < 0.01$) serum sulfate levels than BDDGS or control cattle in both backgrounding and finishing phases. Mean serum sulfate concentrations in cattle fed WDDGS and CDDGS were lower ($P < 0.01$) in finishing phase relative to backgrounding phase despite the higher S intake ($P < 0.01$).

In the metabolic study, effects of dietary S concentration and forage-to-concentrate ratio (F:C) on S and thiamine metabolism, SCFA absorption, and mineral status were evaluated using 16 ruminally cannulated heifers (initial BW 628 ± 48 kg) in a randomized complete block design

with a 2 × 2 factorial treatment arrangement with main effects of dietary S (LS = 0.3% vs. HS = 0.67%, DM) and F:C (Low F:C = 4% vs. High F:C = 51% barley silage, DM). There was no interaction between S concentration and the F:C. The HS cattle had reduced DMI ($P < 0.001$) and SCFA (acetate, propionate and butyrate) absorption ($P < 0.05$) but greater concentrations of ruminal hydrogen sulfide (H_2S) ($P < 0.01$), serum sulfate ($P < 0.01$) and urinary sulfate ($P < 0.01$), as well as greater urinary sulfate excretion ($P < 0.01$) than LS cattle. Free thiamine, thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) concentrations in blood and rumen fluid did not vary ($P > 0.05$) among HS and LS cattle. Concentration of TPP was increased by 9.2% ($P = 0.10$) but with a concomitant numerical decrease in free thiamine in HS brains than LS brains. The HS brains had greater TMP ($P = 0.01$) and total thiamine (free thiamine + TMP + TPP) ($P < 0.01$) than LS brains. The HS cattle had reduced ($P < 0.05$) ruminal cobalt (Co), iron (Fe) and manganese (Mn) but similar ($P > 0.05$) copper (Cu), magnesium (Mg), molybdenum (Mo), selenium (Se) and zinc (Zn) relative to LS cattle. There were reduced serum Mg ($P = 0.003$), Fe ($P = 0.036$) and Mn ($P = 0.100$) concentrations in HS cattle than LS cattle. Brain minerals except for Se did not differ ($P > 0.05$) among HS and LS brains. The F:C did not affect ($P > 0.05$) DMI, S metabolism, blood and brain thiamine and its phosphate esters, and brain mineral status. Ruminal pH, and serum Cu and Se were reduced ($P < 0.05$) and SCFA absorption, ruminal thiamine and its phosphate esters and serum Mg were greater ($P > 0.05$) for low F:C diet. No gross or microscopic changes indicative of PEM were detected in the brains of any experimental heifers.

In field observation, brain thiamine and its phosphate esters, and mineral status were evaluated from 4 naturally occurring S-induced polioencephalomalacia (PEM) affected feedlot

steers. Data from PEM brains were compared with the brains of experimental heifers fed HS diet that were considered normal brains as they had no gross or microscopic changes indicative of PEM. The PEM brains had 36.5% lower TPP ($P < 0.05$) despite the 4.9 fold higher free thiamine ($P < 0.01$), and had reduced Cu ($P = 0.058$), Fe ($P = 0.003$) and Mo ($P < 0.001$) concentrations in comparison with normal brains.

The results indicate that serum sulfate levels in cattle fed DDGS supplemented diets reflect dietary S intake. Sulfur metabolism in beef heifers was not influenced by F:C but high dietary S inhibits SCFA absorption. Moreover, dietary S may increase metabolic demand for TPP. Failing to supply enough TPP may lead to the development of malacic lesions in animals affected by S-induced PEM. Reduced nutritional status of minerals such as Cu, Mo, and Zn associated with excessive S intake were not observed. Sulfur-induced PEM affected cattle exhibited reduced brain Cu, Mo and Fe.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Steve Hendrick for his tremendous support, constructive guidance and endless encouragement throughout my program. I sincerely appreciate for all the patience and help Dr. Hendrick has given me during my study. I would also like to express my special appreciation and thanks to my co-supervisor Dr. John McKinnon, who provided me with constructive guidance, valuable advice, and patience during my studies. Without my supervisors' help and support, this thesis would not have been accomplished. I gratefully acknowledge my advisor committee member Dr. Greg Penner for his special help in designing my metabolic trial, data analysis and interpretation. Special thanks also go to my advisor committee chair Dr. Joe Stookey and my external examiner Dr. Karen Koenig for their time, advice and guidance.

I would like to thank Dr. Andrew Olkowski, who helped me not only to develop important laboratory analytical skills but also to realize the importance of some skills in scientific research. Also without his assistance, my thiamine analysis would not have been such successful. I would also like to thank Dr. Elemir Simko for providing special service in all the pathology work involved in my research project, as well as for his significant contribution to some interesting results contained in my thesis. I wish to thank Dr. Phyllis Shand for providing me with the access to HPLC for my thiamine analysis.

I express my appreciation to the following students for their help with cleaning the barn, feeding cattle, and sampling during my metabolic trial: Hayder Al-Shannon, Marcos Zenobi, Paula Tribulo, Federico Añez, Siping Zhang, Kate Davis, Alin Freidt, Matthew Walpole, Gwinyai Chibisa, Rodrigo Albornoz, Khalil Sahtout, Alvaro Garcia Guerra, Kiran Doranalli,

Christine Rosser, Jocelyn Loos and Sarina Armstrong.

My appreciation also goes to the following funding agencies for their financial support to my research project: Agricultural Biofuels Innovation Program, Alberta Beef Producers, Beef Cattle Research Council, Elanco Animal Health and Agriculture Development Fund. My thanks also go to the staff at the Department of Large Animal Clinical Sciences, University Feedlot and Livestock Research Barn for providing various help during my research work.

I want to thank our department secretaries Jackie and Jennifer for their assistance in ordering supplies for my project. I deeply appreciate Alina at the Toxicology Lab of PDS for her assistance in mineral analysis.

I take the opportunity to also thank the following friends for their care and help during my stay here in Canada. I am sincerely grateful to my best friends Dennis and Marie-Jeanne Will, and Marvin Gennon for their extraordinary kindness to my family and me. I also appreciate Rodney and Heather Gale, Les Kumor and Tyler O'neill for their various aspects of support.

I'm sincerely grateful to my parents, brothers and sisters for their endless encouragement and support, and always standing behind me. My thanks go also to my parents, brother and sister in law for their support during my study.

Last, but not the least, I am extremely indebted to my wife Mikrigul for her endless love and tremendous support throughout my study. Regardless of her being 5-6 months pregnant, she spent days and some nights with me at the barn helping with feeding, cleaning, sampling and preparing buffers during my metabolic trial. I also thank my daughter Subina for bringing more joy and laughter to my life.

DEDICATION

This thesis is dedicated to the following people who are most special in my life:

- My dad Amat Niyaz, and my mom Hansahan Yasin
- My brothers Mamat and Ahat Amat, and my sisters Wurgul and Nurgul Amat
- My wife Mikrigul and my daughter Subina
- My best friends Dennis and Marie-Jeanne Will

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Amino acid
AD	Alzheimer's disease
ADF	Acid detergent fibre
BDDGS	Blend dried distillers grains with solubles
BW	Body weight
Ca	Calcium
CDDGS	Corn dried distillers grains with solubles
Co	Cobalt
CP	Crude protein
Cu	Copper
d	Day
DDGS	Dried distillers grains with solubles
DM	Dry matter
DMI	Dry matter intake
F:C	Forage-to-concentrate ratio
Fe	Iron
h	Hour
H_2S	Hydrogen sulfide
HC	High concentrate
HGHS	High grain high sulfur
HGLS	High grain low sulfur
HPIC	High performance ion chromatography
HPLC	High performance liquid chromatography
HS	High sulfur
HS^-	Hydrosulfide
HSO^{3-}	Hydrogen sulfite

ICP - AES	Inductivity coupled plasma atomic emission spectroscopy
L	Liter
LC	Low concentrate
LGHS	Low grain high sulfur
LGLS	Low grain low sulfur
LS	Low sulfur
M	Mole
MAA	Microbial amino acid
Mg	Magnesium
Min	Minute
ml	Milliliter
m*M*	Millimole
Mn	Manganese
Mo	Molybdenum
Na	Sodium
NDF	Neutral detergent fibre
ng	Nanogram
NRC	National Research Council
P	Phosphorous
PDHC	Pyruvate dehydrogenase complex
PEM	Polioencephalomalacia
ppm	Parts per million
RCB	Randomized complete block
S	Sulfur
S^0	Elemental sulfur
S^{2-}	Sulfide
SCFA	Short chain fatty acid
Se	Selenium

SO_4	Sulfate
SRB	Sulfate reducing bacteria
TCA	Tricarboxylic acid cycle
TK	Transketolase
TMP	Thiamine monophosphate
TMPase	Thiamine monophosphatase
TPK	Pyrophosphokinase
TTP	Thiamine triphosphate
UV	Ultraviolet
VFA	Volatile fatty acid
WDDGS	Wheat dried distillers grains with solubles
wk	Week
WKS	Wernicke-Korsakoff syndrome
WRR	Temporarily isolated and washed reticulo-rumen
Zn	Zinc
α-KGDH	α-ketoglutarate dehydrogenase

1.0 GENERAL INTRODUCTION

Excessive S intake is a common cause of PEM and reduced nutritional status in cattle. Sulfur concentrations in cattle diets often exceed the recommended maximum level (0.4% DM basis) (NRC, 1986) when a high S containing feedstock such as DDGS is included in the diet. Sulfur content of DDGS is considerably higher than that of parent cereal grains owing to the fermentation process (Zhang, 2010). Feeding DDGS is therefore a potential method to induce PEM in cattle (Buckner et al., 2008). This limits the level at which grain can be replaced with DDGS in feedlot rations (Leupp et al., 2009; Neville et al., 2010). Outbreaks of S-induced PEM have occurred in feedlots throughout the world and caused significant economic loss to the cattle industry (Cummings et al., 1995b; Loneragan et al., 1998; Niles et al., 2000; Haydock, 2003; Kul et al., 2006; Knight et al., 2008; Richter et al., 2012). Although S-induced PEM has been recognized since early 1980s (Raisbeck, 1982), the role that S plays in PEM remains unclear (Olkowski, 1997). Inhalation and absorption of eructed H_2S gas from the rumen is proposed to be the major factor inducing PEM (Gould, 1998). However, no conclusive evidence is available to support that the amount of inhaled of eructed ruminal H_2S gas is sufficient enough to cause PEM lesions in cattle (Olkowski, 1997; Niles et al., 2002a). Thus, there is a need to find a diagnostic indicator that identifies the risk of PEM; serum sulfate may serve as an indicator for this disease.

Excessive S intake is also associated with reduced thiamine status in cattle (Goetsch and Owens, 1987; Gooneratne et al., 1989b). High dietary S has potential to induce thiamine deficiency due to destructive effects of sulfites on thiamine molecules (Olkowski et al., 1993b; Alves de Oliveira et al., 1997). Thiamine, on the other hand, is also thought to protect the tissue

from S toxicity by scavenging sulfite or sulfide molecules (Olkowski, 1997). Sulfur-thiamine interaction has been evaluated based on the measurement of total thiamine in biological tissues of cattle, but information on relationship between S and thiamine phosphorylation in beef cattle is limited.

Excessive S intake can reduce nutritional status of some minerals. Excess S may interact with Cu (Gooneratne et al., 1989a; Suttle, 1991), Mo (Spears, 2003), Mg (Ferreira et al., 1966a; Ferreira et al., 1966b; Richter et al., 2012b), Se (Spears, 2003), Fe (Standish and Ammerman, 1971; Suttle, 1974) and Zn (Gooneratne et al., 2011), and influence the absorption and availability of these minerals. Indeed, the interactions between S and other minerals discussed above can reduce S toxicity by rendering the S biologically inactive. Hence evaluating interactions between S and other minerals is necessary to understand both nutritional status of some minerals and S toxicity in cattle fed high dietary S.

An inhibitory effect of S on SCFA metabolism in colonic epithelial cells has been documented (Roediger et al., 1993b; Roediger et al., 1997; Leschelle et al., 2005; Blachier et al., 2009). Sulfides were found to inhibit butyrate and acetate oxidation in colonic epithelial cells (Roediger et al., 1993a; Roediger et al., 1993b; Leschelle et al., 2005). However, the relationship between high dietary S and SCFA absorption from the rumen has not been studied. Since SCFA metabolism in epithelial cells is inhibited by sulfides, it is possible that high dietary S may also influence ruminal absorption of SCFA.

It is suggested that S toxicity may be influenced by the nature of diet (Vanness et al., 2009; Neville et al., 2010). High dietary S in conjunction with high concentrate finishing rations is believed to increase the incidence of PEM in feedlot cattle (Kung et al., 1998). However,

recent research suggests that interactive effects of dietary S and grain may reduce the incidence of PEM (Neville et al., 2010). Sulfide is the form of S that exerts toxicity to the brain tissue and is involved in interacting with other nutrients. Since sulfide, thiamine and mineral status in the rumen are influenced by the composition of the diet, S toxicity and S interactions with other nutrients are expected to be different in cattle fed different F:C. Feeding DDGS based diets may also influence S toxicity and S-nutrient interactions as DDGS contain different forms of S and are rich in minerals and protein but low in starch.

This thesis summarizes data from a small pen study, a metabolism study and a field observation. The effects of feeding DDGS from corn, wheat or a 50:50 corn:wheat blend DDGS on serum sulfate levels of feedlot steers were evaluated in small pen study. Sulfur and thiamine metabolism, mineral status, as well as SCFA absorption in cattle fed high S containing diets with different forage-to-concentrate ratios were evaluated in the metabolic trial. Thiamine and mineral status in the brains of naturally occurring S-induced PEM affected cattle were also evaluated.

Thesis organization: The data from the small pen study, metabolism study and field observation will be present in four different chapters (Chapter 3, 4, 5 and 6). Chapter 3 contains the data from small pen study. Chapter 4 will present partial data from metabolic trial pertaining to the effects of dietary S concentration and F:C on ruminal fermentation, S metabolism and SCFA absorption in beef heifers. The remaining data from the metabolic trial is combined with the data from the field observation and divided into two chapters, Chapter 5 and 6. Chapter 5 and 6 will be focused on the thiamine and mineral data, respectively.

2.0 LITERATURE REVIEW

2.1. Sulfur

2.1.1. General Introduction of Sulfur

Sulfur is one of the abundant elements in living organisms with atomic number of 16. It is present as elemental S, and as organic or inorganic forms in various oxidation status including sulfates, sulfites and sulfides (Olkowski, 1992; Mikus, 2004). Sulfur is an essential component of some amino acids (AA) (cysteine and methionine), vitamins, hormones and sulfated polysaccharides. Cysteine is a precursor for the antioxidant glutathione which protects the cells from oxidative damage (Murakami et al., 2009; Requejo et al., 2010). Methionine is a building block for protein and also serves as a methyl group donor for many transmethylation reactions (Olkowski, 1992). Coenzyme A also contains S. It is an important coenzyme required for the synthesis and oxidation of fatty acids, as well as the oxidation of pyruvate in the tricarboxylic acid (TCA) cycle. In addition, some vitamins such as thiamine and biotin possess S. Thiamine is an important vitamin in cellular energy metabolism. Biotin is essential for fatty acid synthesis and gluconeogenesis. Furthermore, sulfate containing lipids and polysaccharides are incorporated in the structure of the cell wall and connective tissue (Olkowski, 1992). Thus, S is an important nutrient for all living organisms and is required for numerous biochemical processes. Plants can meet their normal S requirement by assimilating simple mineral compounds from the environment, whereas animals must obtain S from the diet (Suttle, 2010).

2.1.2. Sulfur Nutrition in Ruminants

2.1.2.1. Sulfur Requirement

Sulfur is an indispensible mineral for ruminants. It is required by ruminants not only to maintain normal cellular metabolism but also to maintain optimal function and growth of rumen microorganisms (NRC, 2000). Inorganic or organic forms of S in the rumen are utilized by microorganisms and incorporated into S containing compounds such as cystine, cysteine, methionine, thiamine, biotin or other organic compounds. These S containing compounds are essential for ruminal microbial protein synthesis, ruminal fermentation, cellular growth and cellular oxygen transportation. Sulfur deficiency is often associated with depression of microbial protein synthesis, loss of appetite or poor digestibility of fibrous diets (Suttle, 2010). In some cases, S deficiency can result in death of the animal (NRC, 1996). The recommended S level for growing beef cattle is 0.15% (NRC, 1996), and 0.14 - 0.26 % (DM) for sheep (NRC, 2007). Sheep have higher S demand than cattle due to the additional requirement of S for wool growth. However, excess S can adversely impact animal health and production. The maximum tolerable level of dietary S set by the NRC (2000) for both beef and sheep is 0.4 % (DM). However, this level can vary according to the nature of the diet. For cattle eating diets containing more than 40% forage an upper limit has been suggested to be 0.5%, while for the cattle consuming high grain diets (< 15% forage) the upper value is 0.30% (DM) (NRC, 2005).

2.1.2.2. Sulfur Metabolism in the Rumen and Absorption

Sulfur ingested by ruminants occurs in both inorganic and organic forms in nature (Figure 2.1). Normally a variety of S compounds in the rumen are converted into sulfide by

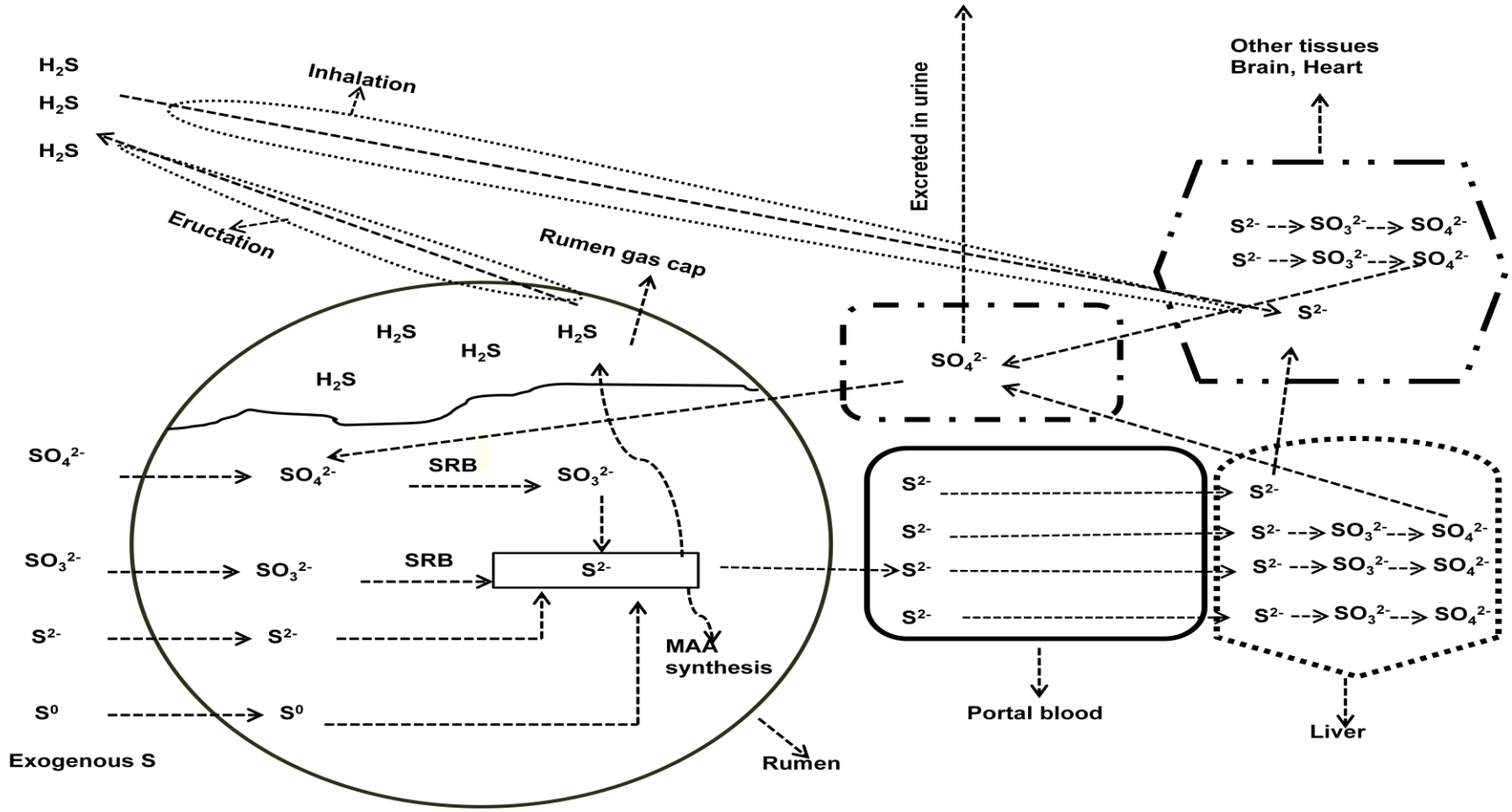


Figure 2.1. Schematic outline of sulfur metabolism in ruminants
(S^0: elemental sulfur; S^{2-} : Sulfide; SO_3^{2-}: sulfite; SO_4^{2-}: sulfate; H_2S: hydrogen sulfide gas; SRB: sulfate reducing bacteria; MAA: Microbial amino acid)

rumen microorganisms (Lewis, 1954; Olkowski, 1992). Two types of sulfate reducing bacteria (SRB) are present in the rumen: assimilatory and dissimilatory SRB. The assimilatory SRB utilize the sulfate to synthesize S containing amino acids namely methionine and cysteine. Whereas, the dissimilatory SRB (i.e. Desulfovibrio and Desulfotomaculum genus) utilize sulfate to maintain their energy needs and produce hydrogen sulfite (HSO_3^-) and H_2S as metabolites (Kung et al., 1998). The SRB are adaptive and increase their activity when high levels of S are ingested and thereby produce excess sulfides in the rumen (Gould et al., 1997; Kung et al., 1998). The S compounds formed after the reduction process by dissimilatory SRB are S_2^-, S^0, HS^-, or HSO_3^- (Kung et al., 1998). Some of these reduced forms of sulfide are protonated and form H_2S and are released into the rumen gas cap, while majority of remaining sulfides in the rumen fluid is either utilized for S containing AA synthesis, or absorbed across the rumen wall. Absorption is considered the major mechanism for sulfide disappearance from the rumen (Bray, 1969). The absorbed sulfide is oxidized to sulphate in the blood and liver. The liver is considered the major organ where most of absorbed sulfide undergoes oxidation. Sulfate is then distributed to excellular fluid, with some sulfate recycled to the rumen through saliva (Gould, 1998). In tissues, sulfide is metabolized through three ways: a) oxidation to sulfate; b) methylation; c) reaction with metallo or disulfide containing proteins (Olkowski, 1992).

2.1.2.3. Sulfur Excretion

Sulfur is excreted from the body via urinary and fecal excretion with majority by the urine. Urinary S is both organic and inorganic and includes sulfate and S containing AA (Suttle, 2010). Urinary S excretion is influenced by S intake (Doyle and Moir, 1979b). It has been

reported that lambs fed 0.84% dietary S (DM basis) excreted 300% more urine, and 480% more S than lambs which ingested 0.22% dietary S (Neville et al., 2011). Sulfur excreted in the feces is largely in indigestible organic S primarily derived from bacterial protein (Bird and Hume, 1971). Fecal S increases with an increase in microbial protein synthesis (Suttle, 2010).

2.2. Thiamine

2.2.1. Thiamine and Its Phosphate Esters

Thiamine, also known as vitamin B1, is an important water-soluble vitamin. Thiamine is a colorless compound with the chemical formula $C_{12}H_{17}N_4OS$. Its molecular structure consists of pyramidine and thiazole rings joined by a methylene bridge (Figure 2.2). The hydroxyethyl side chain of thiamine can be esterified, generating phosphate esters such as TMP, TPP and thiamine triphosphate (TTP) (Bender, 2003).

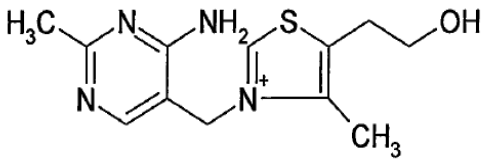


Figure 2.2. Structure of thiamine (McDowell, 2000)

Thiamine is present in mammalian tissues as free thiamine, TMP, TPP, and TTP. Distribution of radioactive thiamine esters in the rat brain was as follows: free thiamine: 8-12%, TMP, 12-14%; TPP, 72-74%, and TTP, 2-3% (Iwata et al., 1985). There is a specific relationship between these forms of thiamine (Figure 2.3). Free thiamine is converted to TPP through phosphorylation. In this process, a pyrophosphate group from ATP is transferred to free thiamine by thiamine pyrophosphokinase (TPK) (Liu and Hurley, 2011). Thiamine pyrophosphate is

further phosphorylated to TPP or dephosphorylated to TMP. Thiamine triphosphate is unstable and rapidly hydrolyzed to TPP. Thiamine monophosphate is hydrolyzed to free thiamine by thiamine monophosphatase (TMPase). (Tallaksen et al., 1992; Butterworth, 2003).

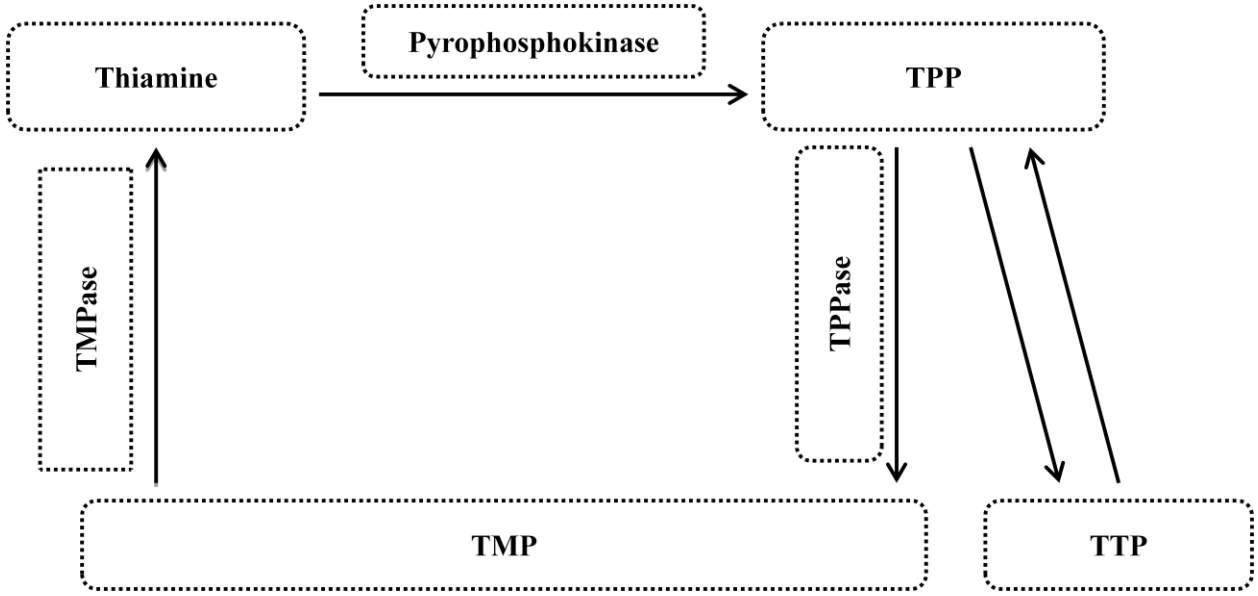


Figure 2.3. The interactions of thiamine, TMP, TPP and TTP in mammalian tissue (Adapted from Tallaksen et al., 1992)

Thiamine monophosphate is an intermediate thiamine ester that facilitates the synthesis of thiamine to TPP. It contains a pyramidine ring that is linked to a thiazole ring, which is in turn connected to a monophosphate group. A specific biochemical role for TMP has not been clearly identified (Heroux et al., 1996; Lonsdale, 2006). It is assumed that TMP might have a role in energy metabolism similar to the stepwise phosphorylation of adenosine (Lonsdale, 2006).

Thiamine pyrophosphate, also called as thiamine diphosphate, is an incredibly active molecule (Lonsdale, 2006). It consists of pyramidine and thiazole rings connected to a

pyrophosphate functional group. The thiazole ring in the TPP molecule is the site that is most commonly involved in reactions. Thiamine pyrophosphate is the only active form of thiamine and plays a fundamental role in energy metabolism. It is a cofactor for three major enzymes including the pyruvate dehydrogenase complex (PDHC), α-ketoglutarate dehydrogenase (α-KGDH), and transketolase (TK) (Lindqvist et al., 1992; Bettendorff et al., 1996; Widmann et al., 2010). The TPP bound as a cofactor to these enzymes is estimated to be 90-95% of the total cellular TPP (Bettendorff et al., 1994).

Thiamine triphosphate is present in most organisms such as fungi, plants and mammalian tissues but the concentration of TTP is relatively low compared to other thiamine phosphate esters. This is likely due to the fact that it is continuously hydrolyzed (Bettendorff et al., 1994). The TTP is generated from the high turnover TPP pool presumably by an ATP-TPP transphosphoralase and is bounded with an unknown TTP binding protein (Bettendorff et al., 1996). The biological function of TTP is involvement in the function of maxi-chloride channels and nerve excitability (Zimatkina et al., 2000).

2.2.2. Brain Disorders Associated With Thiamine Deficiency

Brain is the most vulnerable organ to thiamine deficiency as it relies largely on glucose metabolism to meet its energy requirement. Glucose metabolism is regulated by thiamine dependent enzymes. When thiamine is insufficient, brain glucose metabolism will be inhibited. The inhibition of glucose metabolism in the brain results in a reduction of AA synthesis, diversion of AA from protein synthesis to supply energy via the TCA cycle, decreased lipid synthesis and reduced production of acetylcholine and other neurotransmitters (Thomson et al.,

1983).

Not all the regions of the brain are susceptible to the effects of thiamine deficiency. This is due to: 1) numerous metabolic compartments existing in brain tissue (Thomson et al., 1983), and 2) non-uniform distribution of thiamine esters in different brain regions (Laforenza et al., 1988). Regions with higher metabolic demand are more vulnerable to reduced glucose catabolism and can develop lesions more easily relative to other regions with lower metabolic demand (Thomson et al., 1983). A significant amount of thiamine phosphate esters are present in neurons relative to brain cells. The enzyme activity of thiamine pyrophosphatase is found to be about 20 times higher in the neurons while TMPase is predominately present in glial cells that are non-neuronal cells (Cooper and Pincus, 1979; Laforenza et al., 1988)

The most common neurological disorders associated with thiamine deficiency are Wernicke's encephalopathy, also known as Wernicke-Korsakoff syndrome (WKS) in humans (Butterworth, 2003), and PEM in ruminants (Cebra and Cebra, 2004). Wernicke's encephalopathy is most common in chronic alcoholism and HIV-AIDS victims. It is characterized by selective neuron loss in mammillary bodies, cerebellum, thalamus and pons (Butterworth, 2003). Polioenchephalomalacia is characterized by focal necrosis of the cerebral cortex. Decreased thiamine dependent enzyme activities are considered one of the major factors causing selective neuron loss in these conditions (Butterworth, 2003; Cebra and Cebra, 2004). In addition, suppressed thiamine dependent enzyme activity has been found to facilitate neuron loss in Alzheimer's disease (AD) (Kish et al., 1999) and Parkinson's disease (Mizuno et al., 1994). The former is a progressive degenerative disease of the brain that is often seen in senior people, while the latter is a chronic neurodegenerative disorder that causes people to lose control over

their muscles (Mizuno et al., 1994; Kish et al., 1999).

Reduced activity of thiamine dependent enzymes is primarily caused by a decrease in TPP concentration. This has been studied in both men with WKS and experimentally (Butterworth, 2003). The research conducted to evaluate the relationship between thiamine deficiency and activity of thiamine dependent enzymes, and neuronal loss has been particularly focused on α-KGDH. It has been established that suppressed α-KGDH due to thiamine deficiency results in neuronal death (Butterworth and Heroux, 1989; Butterworth, 2003). A reduction in α-KGDH activity resulting in severe metabolic consequences in the brain is not surprising, because it is a rate limiting enzyme in the TCA cycle. These metabolic consequences are decreased pyruvate oxidation and increased levels of alanine and lactate in the brain (Todd and Butterworth, 1999).

Reduced thiamine dependent enzyme activities have also been found in AD and Parkinson's disease. Decreased concentrations of TPP and a dramatic reduction of TPPase activity (up to 60%) were found in brain tissue of AD patients (Raghavendra Rao et al., 1993; Heroux et al., 1996). The reduced α-KGDH activity in the brains of AD victims has been observed in several studies (Butterworth and Besnard, 1990; Casley et al., 2002). As well, the activities of PDHC (Gibson et al., 1988; Casley et al., 2002) and TK were reduced in AD patients (Gibson et al., 1988; Mastrogiacomo et al., 1996b).

It is unclear if the activities of thiamine dependent enzymes are decreased in the brain of PEM affected animals. It is postulated that reduced thiamine dependent enzyme activity might be the major factor manifesting the neural death in PEM brains (Personal communication with Dr. A.A. Olkowski, University of Saskatchewan, 2012). Thiamine pyrophosphate deficiency appears

to be the primary factor suppressing the activity of thiamine dependent enzymes.

2.2.3. Possible Factors Causing Brain TPP Deficiency

Since insufficiency of TPP is a possible factor associated with a decrease in the activity of thiamine dependent enzymes, it is of importance to discuss the potential factors involved in TPP reduction in brain tissue. The causes of insufficiency of TPP in the brain might be due to the following major factors: 1) thiamine deficient diet; 2) poor absorption and transportation of thiamine; 3) inhibition of TPP synthesis; 4) enhanced TPP degradation (Figure 2.4).

Thiamine pyrophosphate is reported to be decreased due to the inadequate intake of thiamine. Decreased synthesis of TPP was observed in cultured rat cerebral cells exposed to thiamine deficient media (Thornber et al., 1980; Pannunzio et al., 2000). Thornber et al. (1980) found that TPP concentration in the brains of sheep fed a thiamine-free synthetic diet for 4 weeks were reduced by 22% while free thiamine and TMP were found to be reduced to a minor extent relative to TPP reduction. Poor absorption of thiamine from the gastrointestinal tract and the loss of liver thiamine stores due to some liver disease may also contribute to TPP deficiency in the brain (Butterworth, 2003).

Inhibition of TPP synthesis from free thiamine could be a major contributor to TPP deficiency in the brain. Thiamine pyrophosphate is synthesised from free thiamine. This phosphorylation process requires enough free thiamine, ATP, Mg^{2+}, as well as normal function of TPK. The inhibition of TPP synthesis occurs when any one of Mg^{2+}, ATP and free thiamine is insufficient or the enzyme activity of TPK is inhibited. Mastrogiacomo et al. (1996a) observed

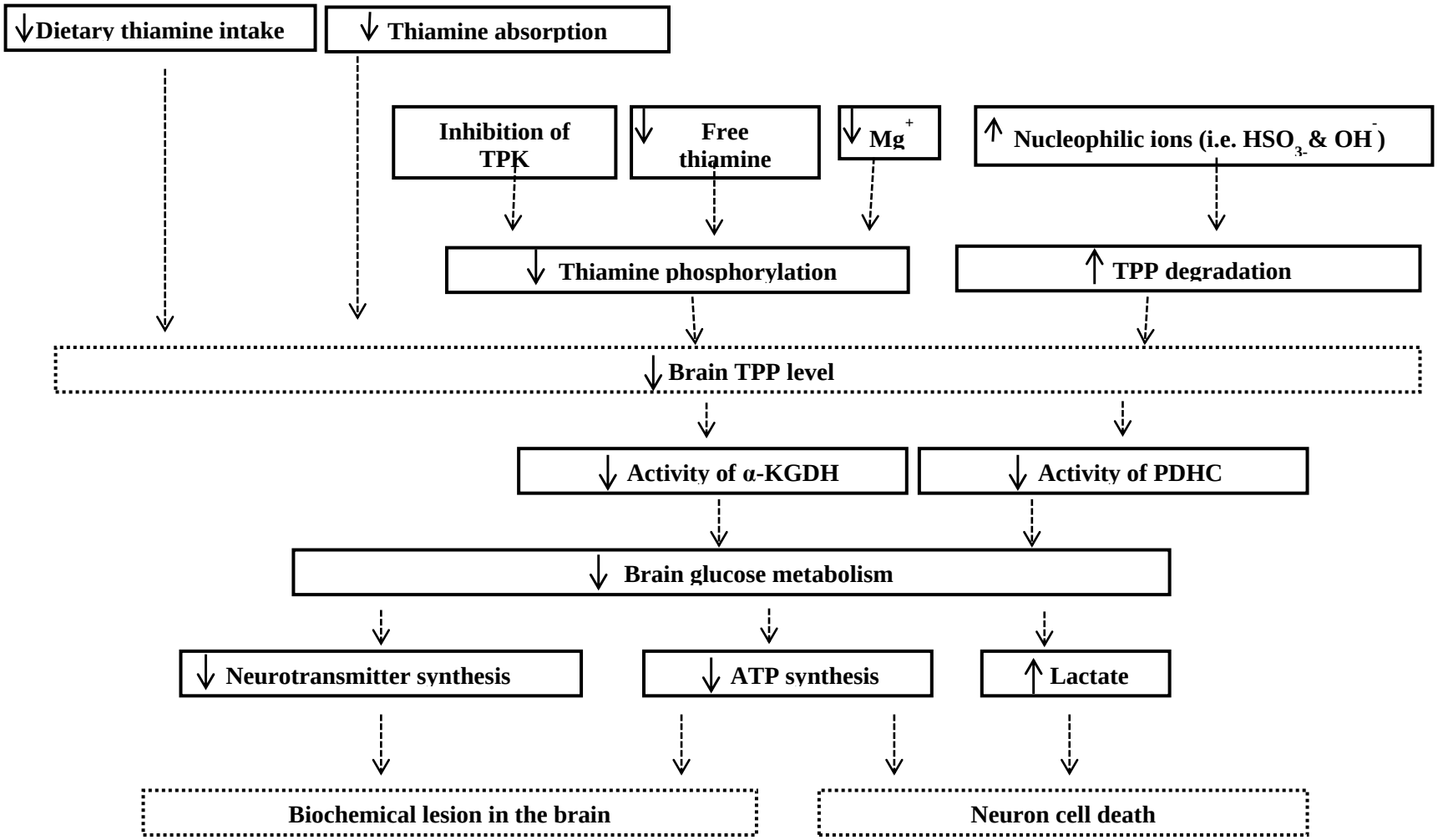


Figure 2.4. Proposed mechanism of brain disorders associated with thiamine deficiency (Adapted from Butterworth, 2003)

that TPP was significantly reduced by 18-21% while free thiamine and TMP were remained unaltered in the brain of AD patients. Since ATP levels in the brains of AD patients are reduced, they proposed that this TPP reduction was due to the reduction of the TPK activity as TPK is an ATP dependent enzyme. Raghavendra Rao et al. (1993) also reported that there was a 60% decrease in TPK activity in the brain of an AD patient that had decreased TPP. TPP synthesis cannot be performed when there is not enough Mg^{2+}. This results in an apparent thiamine deficiency, even when the body has enough or excess thiamine (Johnson, 2001).

Enhanced degradation of TPP could be another major factor that causes insufficiency of TPP in the brain. Some factors such as nitrates and nucleophilic reagents may induce cellular TPP degradation. Since TPP is a very active molecule, it is more readily degraded by sulfite (HSO_3^-) which is a strong nucleophile. Hydroxyl free radicals (OH^-) can also degrade TPP (Belitz et al., 2009). Thiamine pyruphosphate may also be deactivated by nitrates that can react with the amino group of the pyrimidine ring of the TPP molecule (Belitz et al., 2009). It has also been shown in vitro that the Cu, Mo and Fe could increase the degradation of TPP. Farrer (1947) observed the effect of Cu on the rate of thiamine destruction in phosphate buffer solutions in vitro. He found that thiamine was destroyed more rapidly in the presence of Cu than in its absence. Farrer (1947) also suggested that other heavy metals such as Fe, Zn in phosphate nitrate solutions could accelerate thiamine degradation. The effects of these heavy metals on thiamine degradation have not been studied in vivo. Furthermore, α-KGDH enzymes are reduced in the brain of AD patient and the reduction of these enzymes is postulated to be involved in the decomposition of TPP.

The α-KGDH enzyme is acting as a “sink” to its cofactor TPP. When this protein is

reduced, the affinity of TPP for its apoenzyme would be diminished; unbound TPP will thereby be easily converted or hydrolyzed to TMP by thiamine pyrophosphatase (Kish et al., 1999; Butterworth, 2003).

2.2.3.1. Thiamine Absorption, Distribution and Excretion

The rumen is not considered as the site for thiamine absorption as the rumen wall mucosa has an extremely low permeability to thiamine (Hoeller et al., 1977). Thiamine is mainly absorbed from the small intestine (Miller et al., 1986). The majority of absorbed thiamine is microbial thiamine. Breves et al. (1980) observed that 90-96% of the thiamine entering the intestinal is microbial origin and is in the free form. Intestinal uptake of thiamine is accomplished by a dual mechanism: a carrier–mediated saturable process and a non-saturable diffusion process (Harmeyer and Kollenkirchen, 1989; Rindi and Laforenza, 2000). The uptake of thiamine from the intestine is a temperature, energy dependent, pH sensitive, and Na^+ independed process. It is inhibited by thiamine structural analogs such as amprolium and oxythiamine, as well as amiloride (Said et al., 1999). The entry of thiamine into the enterocyte takes place after the translocation of thiamine to the intravascular space. This process is Na+ and biotransformation independent and is completely inhibited by thiamine analogs. The thiamine transport from the enterocyte to portal blood is a Na^+ dependent process and directly coupled to ATP hydrolysis by Na^+-K^+-ATPpase (Rindi and Laforenza, 2000).

In the blood, thiamine is found both intra and extracellularly. Greatest level of thiamine is in extracellular. Whole blood thiamine is assumed to reflect both cellular and extracellular thiamine, thus it is often used to monitor thiamine deficiency. All forms of thiamine are present in the blood (Tallaksen et al., 1992; Tallaksen et al., 1993). Thiamine pyrophosphate accounts

for 80% of total blood thiamine (Tallaksen et al., 1992).

Despite the fact that thiamine is readily absorbed and transported to body tissues, thiamine is not stored to a great extent in organs and it has to be continuously supplied. Thiamine distribution in different organs varies considerably. Brain, liver, heart and kidney have higher thiamine concentrations than muscle tissue (Tanphaichair, 1976). Since thiamine is one of the most poorly stored vitamins, body stores in animals fed thiamine-deficient diets will be exhausted within a couple weeks (Ensminger et al., 1990).

Thiamine is excreted via urine and feces. Fecal thiamine originates from the unabsorbed thiamine and endogenously excreted thiamine. Urinary excretion is the dominant avenue for thiamine excretion (Olkowski, 1992). It has also been identified that some thiamine is excreted in the bile and intestinal mucosa (Braunlich and Zintzen, 1976).

2.3. Sulfur-Thiamine Interaction

2.3.1. Mechanism of Sulfur-Thiamine Interaction

An interaction between excess dietary S and thiamine in ruminants is evident. Goetsch and Owens (1987) observed that high dietary S reduced the amount of thiamine passing from the rumen in dairy steers. Gooneratne et al. (1989b) also found S-thiamine interaction in a PEM affected cow. This animal exhibited thiamine deficiency due to a high intake of S containing water. The effect of excess S on blood thiamine in beef cattle was evaluated in a study by Olkowski et al. (1991), who concluded that high S containing diets may have detrimental effects on blood thiamine. Following this finding, they investigated the thiamine–destroying activity of S in native or thiamine-spiked cultures. They found that thiamine destroying activity in rumen

contents was increased by sulfate. No effect of sulfate on the thiamine synthesis was observed (Olkowski et al., 1993a). The adverse effects of high S on net thiamine production was observed in an in vitro study in which a semi-continuous culture system was used to evaluate the effects of sulfate on thiamine synthesis (Alves de Oliveira et al., 1997).

The detrimental effects of high S on thiamine may result from the fact that sulfite can cleave thiamine (Williams et al., 1935). Thiamine molecule is usually split by sulfite into biologically inactive compounds namely sulfonic acid and thiozole (Williams et al., 1935). The rate of thiamine cleavage is influenced by several factors including temperature, pH, and concentrations of either thiamine or sulfite. The thiamine cleavage reaction is most active at high sulfite concentration, low pH values, or high temperature (Leichter and Joslyn, 1969).

Sulfite is a key intermediate in the reduction of sulfate to sulfide in the rumen and oxidation of sulfide to sulfate in the tissue (Olkowski, 1997). Sulfate entering the rumen is reduced to sulfite and then further reduced to sulfide by SRB (Lewis, 1954). In the tissue, absorbed sulfide is oxidized to sulfite and then to sulfate. A certain portion of sulfate is recycled to the rumen where it is again converted to sulfite and then to sulfide. This recycling process has the potential to maintain a constant level of sulfite in both rumen and tissues (Olkowski, 1997). Hence it is possible that there is enough concentration of sulfite that can exert an adverse effect of thiamine in the rumen and tissues.

2.3.2. Possible Factors Influencing the Sulfur-Thiamine Interaction

Copper may influence the interaction between thiamine and S, and thereby reduce the detrimental effect of S on thiamine metabolism. Gooneratne et al. (1989a) reported that there was a significant increase in blood thiamine concentration in cattle exposed to high S after the

supplementation of Cu. The interaction between Cu and thiamine has been further confirmed by Olkowski et al.,(1991). They observed that sheep fed a high S containing diet with a high Cu supplement had higher blood thiamine but lower liver thiamine relative to sheep with a low Cu supplement. The interaction between Cu and thiamine may be important in reducing the detrimental effects of S on thiamine metabolism (Olkowski et al., 1991). Olkowski et al. (1992) suggested that Cu could protect the thiamine molecule from the destructive effect of sulfite by making a conformational change to the thiazole open ring.

2.3.3. Effects of Sulfur on Thiamine Phosphate Esters and Thiamine Dependent Enzymes

The detrimental effects of S on thiamine phosphate esters have also been reported. Lenz and Holzer (1985) reported that free thiamine, TMP and TPP in yeast (saccharomyces cerevisiae) were cleaved by sulfite. Sulfite could also reduce cellular TPP via either inhibiting the synthesis or enhancing degradation or both. Sulfite is reported to be involved in the degradation of TPP as it is a very active molecule (Belitz et al., 2009). In addition, sulfite is more likely to inhibit TPP synthesis from free thiamine by inhibiting ATP production that is required by TPK. Increased degradation and/reduced TPP synthesis subsequently leads to changes in the activity of thiamine dependent enzymes. A study by Lenz and Holzer (1985) supports this point. They found that two TPP dependent enzymes, α-KGDH and TK, were inactivated by 5 m*M* sulfite within 1 h to 58% and 13% of the initial values, respectively. This enzyme inactivation corresponded with a 36 % reduction in the intracellular TPP. To date, however, the detrimental effect of high dietary S on thiamine dependent enzyme activity in ruminant or monogastric animals has not been investigated.

2.4. Sulfur-Mineral Interactions

It has been documented that S directly interacts with other minerals such as Cu, Mo (Olkowski, 1992), Se (Van Ryssen et al., 1998) and Mg (Richter et al., 2012). While the interactions between S and other minerals have the potential to cause a deficiency, these interactions can also render S biologically inactive and thereby reduce S toxicity (Mikus, 2004). Therefore, it is important to consider sulfur-mineral interactions in animals fed high dietary S.

2.4.1. Sulfur-Copper-Molybdenum Interactions

Interactions of S with Cu and Mo in ruminants have been intensively studied (Suttle, 1975; Gooneratne et al., 1989a; Richter et al., 2012). Interactions between these three minerals occur during the process of digestion and absorption, and systemically, which results in forming an insoluble complex called thiomolybdates (Gooneratne et al., 1989a; Suttle, 1991). Because of these interactions, animals on higher dietary S often suffer from Cu deficiency which becomes more intense when excessive Mo is present (Van Ryssen et al., 1998). In addition, S can reduce Cu bioavailability by forming an insoluble CuS complex (Suttle, 1974). Several studies reported Cu deficiency in cattle exposed to high dietary S. Smart et al. (1986) observed that cattle exposed to high S containing water exhibited reduced liver Cu. Gooneratne et al. (1989b) found that one cow affected by S induced PEM exhibited Cu deficiency. Recent studies done by Richter et al. (2012) showed that plasma and liver Cu levels were significantly reduced in the beef steers fed 0.6% dietary S relative to those fed 0.3% dietary S.

An interaction between S and Mo, independent of Cu may also exist. It has been reported that Mo interferes with sulfate reduction to sulphide (Gawthorne and Nader, 1976). In contrast,

Mills (1960) found that high dietary Mo increased the sulfide concentrations in the rumen of sheep. Huisingh et al. (1975) identified that dietary sodium molybdate significantly reduced sulfide production from sulfate. Sulfate reduction was also shown to be inhibited due to the inhibition of human gut SRB by sodium molybdate (Willis et al., 1997). Considering the above, S status in ruminants is likely to be influenced by high dietary Mo via either forming thiomolybdate or inhibition of SRB or both.

2.4.2. Sulfur-Magnesium Interaction

In ruminants, high S can interact with Mg and make it biologically less available. The interaction between S and Mg in ruminants was first reported by Spears et al. (1985). They observed that steers fed forages that were fertilized with S showed a reduction in Mg absorption. In addition, Richter et al. (2012) reported that plasma Mg concentration of backgrounding steers was decreased as dietary S increased, while no effect was observed at the finishing stage. They postulated that the reason why the effects of S on Mg occurred while cattle consumed a high forage diet may be due to the favourable rumen conditions for the formation of insoluble complex between S and Mg. High S inhibits the absorption of Mg across the rumen wall. In vitro and in vivo studies conducted in 1960s showed that substitution of Cl^- with SO_4 ions results in an increased transmural potential difference which results in reduction of Mg absorption (Ferreira et al., 1966a; Ferreira et al., 1966b). Furthermore, Martens and Blume (1986) reported that in vitro, sulfate is a more potent inhibitor of Mg uptake across the rumen epithelium than Cl^- due to a rise in lumen pH.

2.4.3. Sulfur-Selenium Interaction

Few studies have evaluated the interaction between S and Se. Interaction between S and Se was first reported by Whanger et al. (1969). They observed that the incidence of white muscle disease in lambs was increased as sulfate supplementation increased. Later the interaction between S and Se was confirmed (White and Somers, 1977; Pope et al., 1979; Pope et al., 1979). Van Ryssen et al. (1998) investigated the effect of concentration of S in a diet on the interrelationship between Cu and Se in sheep. They found that the Se concentration was reduced by the addition of S. This reduction was more aggressive at high Cu intake resulting in a significant three-way interaction between these three minerals. They suggested that the antagonistic effects of dietary S on the metabolism of both Cu and Se could contribute to the occurrence of an interrelationship between the two minerals and that the concentration of S in the diet should be considered when evaluating such an interaction. These authors concluded that an increase in S intake decreased the accumulation of both Cu and Se in the liver of sheep, reducing the interaction between Cu and Se.

2.5. Polioencephalomalacia

Polioencephalomalacia is an important neurological disorder that can affect many species of ruminants all over the world and cause significant economic loss (De Sant'Ana and Barros, 2010). It was first reported in North America by Jensen et al. (1956). This disease is characterized by a focal necrosis of cerebral cortex. The primary lesions are limited to the brain (Roberts and Boyd, 1974). Polioencephalomalacia is the softening (malacia) of the gray matter (polio) of the brain (encephalo). Animals at all ages can be affected but young animals seem to

be more vulnerable (Niles et al., 2002a; Rachid et al., 2011). Affected animals frequently respond to thiamine administration (Rammell and Hill, 1986; Olkowski, 1997; Gould, 2000). Polioencephalomalacia has been associated with thiamine deficiency, S toxicity, lead toxicity, and water deprivation-sodium ion toxicity. All these etiological factors produce similar brain lesions (Gould, 1998; Niles et al., 2002a). Thiamine deficiency and S toxicity have been recognized as the major causes of PEM.

2.5.1. Thiamine Deficiency Induced PEM

Polioencephalomalacia associated with thiamine deficiency has been reported in cattle, sheep, horses, dogs (Rammell and Hill, 1986), goats (Sakhaee and Derakhshanfar, 2010), camels (Milad and Ridha, 2009), cats (Palus et al., 2010), and wild life (Rammell and Hill, 1986). Thiamine deficiency is not commonly observed in ruminant animals and there is no dietary recommendation for thiamine because microbial synthesis is generally considered adequate. Thiamine deficiency in ruminants can be caused by several factors such as an impairment of microbial thiamine synthesis, thiamine destroying activity of bacterial thiaminase and some other dietary factors involved in thiamine destroying activity in the rumen (Brent and Bartley, 1984). Bacterial thiaminase has been considered the main factor causing thiamine deficiency in ruminants. Two types of thiaminase namely type I and II are produced by different types of bacteria in the rumen (Cebra and Cebra, 2004). Both types of enzyme may have a destructive effect on thiamine in the rumen. Thiaminase type I is involved in catalyzing the nucleophilic displacement of the thiazole moiety of thiamine by another base known as a co-substrate and generates thiamine analogues that inhibit thiamine dependent reactions. Thiaminase type I

requires a co-factor to accomplish its thiamine destroying activity. Some medications such as promazines and levamisole, and substrates produced during fermentation appear to be act as cofactor to thiaminase I (Cebra and Cebra, 2004). Thiaminase type II splits thiamine by catalyzing the hydrolysis process and thereby reduces the amount of thiamine absorbed from rumen (Murata, 1982). Several outbreaks of PEM in sheep and cattle caused by high thiaminase activity in the rumen have been reported (Edwin and Jackman, 1970; Roberts and Boyd, 1974). Thiaminase I is also present in plants such as bracken fern, horse tail, and nar do ferns (Rachid et al., 2011). Animals exposed to these plants also developed PEM (Ramos et al., 2003; Ramos et al., 2005).

Thiaminase activity seems to be influenced by the nature of the diet. High concentrate diets may increase the thiaminase type I activity by providing optimum ruminal pH (Brent, 1976). It has been reported that lambs fed highly fermentable diets died of PEM associated with thiamine deficiency induced by high thiaminase type I activity. In addition, rumen acidosis has been reported to precede PEM (Brent, 1976).

Amprolium, a potent coccidiostat and thiamine analogue, is believed to be another major factor that can induce PEM. Amprolium inhibits the conversion of free thiamine to TPP and thereby deprives the tissues (especially brain) of TPP (Loew and Dunlop, 1972; Cebra and Cebra, 2004). Thornber et al. (1979) induced PEM in lambs by feeding a thiamine free diet with high levels of amprolium (280 mg/kg of BW). As well, oral administration of amprolium caused a reduction of blood and tissue thiamine levels and resulted in the development of PEM in calves (Kasahara et al., 1989). However, clinical and histopathological lesions indicative of thiamine

deficiency has been produced in preruminant lambs by feeding a thiamine free artificial milk diet (Thornber et al., 1980). These researchers questioned the hypothesis that the amprolium could be the major factor causing PEM.

Apart from amprolium, other factors such as production of inactive forms or poorly absorbed forms of thiamine in the rumen, inhibition of phosphorylation and absorption could also cause functional thiamine deficiency (TPP deficiency) which subsequently leads to the development of malacic lesions (Cebra and Cebra, 2004). In these circumstances, the free thiamine levels of blood or brain tissue usually appear to be within the normal range of animals or even elevated. Therefore, diagnosing thiamine deficiency according to the blood or tissue free thiamine levels is difficult.

2.5.2. Sulfur-induced PEM

Sulfur toxicity has become increasingly accepted as a major cause of PEM. Outbreaks of S-induced PEM have been reported with an increasing frequency throughout the world in the last three decades. The hypothesis regarding high dietary S associated PEM was first proposed by Raisbeck (1982). This hypothesis was further supported by research from University of Saskatchewan and Colorado State University (Gooneratne et al., 1989b; Gould et al., 1991). Gooneratne et al., (1989a) experimentally developed PEM in sheep by feeding a diet containing 0.63% S. Gould et al. (1991) also induced PEM in Holstein steers by feeding an experimental diet with added $NaSO_4$. Field cases of PEM associated with high dietary S have occurred in feedlots across the world. Kul et al. (2006) reported that 296 out of 5050 dairy and beef cattle were clinically affected by the S-induced PEM. These cattle were fed 0.45% dietary S which

was derived primarily from barley malt sprouts (0.72% S). Knight et al. (2008) reported that 19 steers developed PEM after consuming a high S containing water (> 3000 ppm S). There are numerous other reports regarding dietary S levels arranging from 0.45% to 0.6% that caused clinical and experimental PEM (Cummings et al., 1995a; Loneragan et al., 1998; Niles et al., 2000; Haydock, 2003; Knight et al., 2008; Richter et al., 2012). Although S-induced PEM has been recognized in the last three decades, the role that S plays in PEM remains unclear (Olkowski, 1997).

2.5.2.1. Proposed Mechanisms of Sulfur-induced PEM

Current available dogma for the S-induced PEM is that the inhalation and absorption of eructated H_2S from the rumen is the major contributor to the pathology (Gould, 1998). When excess S is ingested, a relatively high amount of sulfide is being generated as a result of S reduction by the rumen microbes (Figure 2.1). Some sulfide from the fluid phase is released into rumen gas cap as H_2S. Formation of H_2S from sulfide ion is pH dependent. As rumen pH drops, the H_2S in the rumen gas cap will increase. Ruminants inhale 70-80% of the eructed gas (Dougherty and Cook, 1962). Therefore it is proposed that most of the eructed H_2S gas gets into the blood stream in the lungs via inhalation of eructed gas. The majority of the inhaled H_2S can reach the brain without undergoing detoxification mechanism in the liver and thus can exert its toxic effect to the brain (Gould, 1998).

Sulfide in brain tissue is detoxified to sulfate via the mitochondrial sulfide oxidation process (Powell and Somero, 1986). Sulfide oxidation is linked to the respiratory electron transport chain. The electrons from oxidation enter the respiratory chain at the level of

cytochrome c. Mitochondrial sulfide oxidation is inhibited by high sulfide concentrations (O'Brien and Vetter, 1990). When sulfide concentration exceeds a certain level, the cytochrome c oxidase, the last enzyme in the respiratory electron transport chain of mitochondria, will be inhibited. As a result, ATP production through oxidative phosphorylation will be blocked (Smith and Keppler, 1977). This is considered the primary mechanism for sulfide toxicity. Therefore, tissues such as brain that have a high oxygen demand are more sensitive to disruption of oxidative metabolism by sulfide (Ammann, 1986).

Measuring ruminal H_2S gas has been used to identify animals at potential risk of S induced PEM. Gould et al. (1997) suggested that rumen gas cap H_2S concentrations greater than 1000 ppm are potentially toxic and over 2000 ppm can precede the development of PEM. Sulfur-induced PEM affected ruminants showed a variety H_2S concentration ranging from less than 200 (Simko et al., University of Saskatchewan, unpublished data) up to 25000 ppm (Richter et al., 2012). Recent studies showed that ruminants exposed to elevated dietary S (0.65% or 0.83% DM) exhibited relatively high H_2S gas ranging from 2000 to 8000 ppm, but did not show any clinical signs of PEM (Neville et al., 2010). Gould et al. (1997) reported that the cattle with clinical signs of PEM had lower ruminal H_2S than those clinically normal steers (H_2S > 2000 ppm). Loneragan et al. (1998) also found lower ruminal H_2S in a PEM affected calf (450 ppm). Richter et al., (2012) reported that two yearlings steers developed clinical signs of PEM and died due to high dietary S intake (0.5% S, DM). One had only 1000 ppm ruminal H_2S. Unpublished data from the University of Saskatchewan indicated that heifers with S-induced PEM exhibited lower ruminal H_2S concentrations ($\leq$ 200 ppm) than those clinically normal cattle (2000-3600 ppm) (Simko et al., University of Saskatchewan, unpublished data). These data suggested that

H_2S may not be good indicator for assessing the risk of PEM, although lowered H_2S in clinically affected animals may partially be due to anorexia.

Hydrogen sulfide toxicity to the brain is seen in man and laboratory animals. As well, PEM incidences in cattle have been caused by direct inhalation of H_2S from the poison gas wells and manure slurry pits (Hooser et al., 2000). Thus, there is no doubt that inhalation of excess H_2S can cause neurological disorders. However, to date, there is no conclusive evidence to support the theory that the concentration of inhaled H_2S from the rumen is high enough to induce PEM lesions in the brain of ruminants. Olkowski (1997) argued that the concentration of H_2S generated in the rumen of animals exposed to moderate S may not be sufficient to exert acute toxic effects to the brain. In addition, inhalation of eructed H_2S is reported to cause lung tissue damage (Dougherty and Cook, 1962; Gould et al., 1997). However, Niles et al. (2002b) failed to observe any clinical or post-mortem signs of lung damage in calves exposed to high dietary S and had ruminal H_2S concentrations reaching 24,000 ppm. Furthermore, they performed a breath analysis of expired air on calves in the same study and measured H_2S. Surprisingly, they didn't find any detectable amount of H_2S from the expired air of the calves. Taken all together, it is questionable whether H_2S is the major pathogenesis of S induced PEM.

2.5.2.2. Possible Mechanism of Sulfur-induced PEM

Sulfite, another toxic form of S, is also postulated to be involved in the development of S-induced PEM (Olkowski, 1997). Sulfite is a strong nucleophile and can react with wide variety of hormonal and cellular compounds to cause toxicity (Chiarani et al., 2008). The neurotoxic effects of sulfite have been increasingly recognized (Reist et al., 1998; Chiarani et al., 2008). One electron sulfite oxidation is thought to produce sulfite radicals that could damage DNA,

lipids and proteins (Zhang et al., 2004). Chiarani et al. (2008) found that sulfite increased lipid peroxidation and decreased antioxidant enzyme defences in rat brain. In addition, when rat and mouse neuronal cells were exposed to sulfites in vitro, there was an increase in the production of reactive oxygen species and a reduction in intracellular ATP production (Zhang et al., 2004). They also found that glutamate dehydrogenase in the rat brain was inhibited by sulfites; hypothesizing that this might result in energy deficit at the neurons, with secondary inhibition of the TCA cycle (Zhang et al., 2004).

The destructive effects of sulfite on thiamine, and its functional form could be another way that sulfite produces neurotic lesions in the brain. Sulfite can cleave thiamine rendering it biologically inactive. Sulfite is also reported to be involved in degradation of TPP (Belitz et al., 2009). Metabolic deficiency of TPP is associated with neurodegeneration (Butterworth, 2003). It is also possible that the adverse effects of sulfite on mitochondrial ATP production (Zhang et al., 2004) might indirectly inhibit TPP synthesis from free thiamine, as TPP synthesise requires ATP.

Sulfite toxicity in man and laboratory animals has been extensively investigated, whereas this subject in ruminants has attracted little attention. This might partially be due to the fact that more attention has been placed on sulfide toxicity in ruminants due to the fact that sulfite concentrations in the rumen and tissues are believed to be relatively low. However, there is a potential to maintain a constant amount of sulfite in both rumen and tissues due to sulfate reduction in the rumen, sulfide oxidation in the tissue as well as sulfate recycling to the rumen (Olkowski, 1997). Thus, it is possible for sulfite to contribute to toxicity via various pathways discussed above and play a certain role in the pathogenesis of S-induced malacic lesions in brain.

2.5.2.3. Clinical Signs, Gross and Microscopic Changes in Brain of Sulfur-induced PEM

Bothe sub-acute and acute S-induced PEM are recognized (Gould, 2000). Animals affected with the acute form usually develop clinical signs including blindness, recumbency, seizures and eventually death. The symptoms in most cases appear after 10 days of exposure to high dietary S (Cummings et al., 1995a). Animals affected with the subacute form show signs of ataxia, visual impairment and muscle contraction, head pressing and circling. Neurological impairment seen in the subacute form is usually not severe and with animals frequently recovering; however, in some cases this mild impairment may progress and become more severe with the lesions similar to the acute form (Gould, 2000; Niles et al., 2002a).

Typical gross brain lesions of S-induced PEM are brain swelling, softening with gyral flattening, and cerebellar coning resulting from herniation of the medulla and cerebellum into the foramen magnum. Yellowish brown discoloration may also be seen. At the early stage, grossly apparent autofluorescent bands of necrotic cerebral cortex are present under ultraviolet illumination. As the pathogenic process progresses, grossly evident cavitation forms and autoflureescence disappears. In some cases, especially when animals experience severe S toxicity, a deep multifocal cerebral vascular degeneration, haemorrhage and necrosis are present in addition to cerebrocortical neuron necrosis. The chance of recovery is very slim and animals often die (Gould, 2000).

2.5.2.4. Diagnosis of Sulfur-induced PEM

Since neurological lesions caused by S toxicity, thiamine deficiency, lead toxicity and

water deprivation-sodium ion toxicity are similar, one first has to be rule out other possible factors before diagnosing S-induced PEM. Sulfur-induced PEM is only diagnosed through the combination of three assessments: close observation of the clinical symptoms, histopathological examination of the affected brain and assessing total S intake from diet and water. Measuring ruminal H_2S in healthy herd mates of clinically PEM affected animals is reported to be helpful in confirming S-induced PEM (Niles et al., 2002a). Dietary S exceeding 0.4% (DM) (NRC, 1986), water sulfate higher than 1000 ppm (Olkowski, 2009), as well as ruminal H_2S greater than 1000 ppm (Gould et al., 1997) are considered as suggestive toxic levels that can precede the development of PEM (Cebra and Cebra, 2004). So far no effective method is available to diagnose S-induced PEM. Finding an effective means to diagnose S-induced PEM is important.

2.5.2.5. Treatment of Sulfur-induced PEM

Like thiamine deficiency and lead toxicity associated PEM, S-induced PEM can be treated with thiamine. Affected animals should be given a high dose of thiamine (10-20 mg/kg) via intravenous injection (Niles et al., 2002a). The treatment is preferably provided every 6 hours as the thiamine is water soluble and excreted quickly from the body (Cebra and Cebra, 2004). The efficacy of thiamine treatment is more likely to be dependent on the severity of the malacic lesions. Some animals especially those at the early stage of the disease are more likely to respond to thiamine treatment and the improvement can be seen within a few hours of treatment. In some cases, however, the affected animals only respond to prolonged treatment. While in some cases, thiamine treatment is totally ineffective (Olkowski, 1997). The ineffectiveness of the treatment to some animals is presumably due to the deficit in metabolism of thiamine (Personal communication with Dr.A.A, Olkowski, University of Saskatchewan, 2012).

The mechanism underlying thiamine treatment of S-induced PEM remains a mystery. The therapeutic role of thiamine may be due to possible tissue protective function of thiamine from sulfite or sulfite toxicity (Olkowski, 1997). Thiamine molecules are reported to scavenge free radicals (i.e. sulfite ions). In addition, the interaction between thiamine and sulfite that was discussed earlier could be another process to reduce the affects of sulfite toxicity. Considering these mechanisms, it is possible that the brain can be protected by the presence of excess thiamine.

2.6. Effects of Sulfur on Short Chain Fatty Acid Absorption

Short chain fatty acids are not only important energetic and nutritional contributors to the body, but also essential nutrients for maintaining normal epithelial cell growth and absorptive functions of the gastrointestinal (GI) tract (Bergman, 1990). They are produced through the bacterial fermentation of carbohydrates in the GI tract. Acetate, butyrate and propionate are predominant SCFA produced in the GI tract (Bergman, 1990; Aluwong et al., 2010). Production of SCFA varies in different sites of the GI tract. The rumen is the major site for SCFA production relative to other parts of the ruminant GI tract. The majority of SCFA ($\geq$ 90%) are present as ions rather than free acids in the rumen due to ruminal pH (Bergman, 1990). Acetate is present in largest concentration while substantial amounts of butyrate and propionate are also present in the rumen. The typical molar ratio of acetate to propionate to butyrate is reported to be 65: 20: 15 in the rumen (Bergman, 1990). However, concentrations of these SCFA are largely diet dependant (Bergman, 1990; Aluwong et al., 2010). Short chain fatty acids are readily absorbed from the GI tract. The rumen is the major site for SCFA absorption accounting for 88% of total absorbed SCFA (Bergman, 1990). Absorption rate of different SCFA from the

rumen is different and depends on the chain length of SCFA. Absorption rate decreases with a decrease in chain length (butyrate > propionate > acetate). Absorption of SCFA from the rumen is accomplished through passive diffusion and bicarbonate dependent transport (Penner and Aschenbech, 2011). Absorbed SCFA are metabolized in ruminal epithelium cells and provide the majority of the energy required by epithelial cell metabolism. Butyrate is the major fuel source for epithelial cells followed by propionate and acetate. About 90% of butyrate, 50 % of propionate and 30% of acetate are metabolized by ruminal epithelium (Bergman, 1990).

Since SCFA metabolism in epithelial cells are important in maintaining normal growth and optimal function of epithelial cells, any disruption in absorption and/or metabolism of SCFA in epithelial cells will lead to malfunction of epithelial cells. This poses a threat not only to gut health but also to whole body health. Factors such as electrochemical gradients of anions, chemical gradient of undissociated acid, and relative permeability of the epithelium to the anion and acid form, as well as rate of SCFA metabolism by epithelial tissue can affect the absorption of SCFA across the rumen epithelium (Stevens and Stettler, 1966).

The inhibitory effect of S on SCFA metabolism in colonic epithelial cells has been documented (Moore et al., 1997; Roediger et al., 1997; Leschelle et al., 2005; Blachier et al., 2009). Sulfides are found to inhibit butyrate and acetate oxidation in epithelial cells. Inhibition of butyrate oxidation by sulfides in human and rat colonic epithelial cells has been identified in several studies (Roediger et al., 1993a; Roediger et al., 1993b; Moore et al., 1997; Moore et al., 1997; Jørgensen and Mortensen, 2001; Leschelle et al., 2005). In addition, it has been shown that sulfides can inhibit acetate oxidation in human colonic epithelial cells (Leschelle et al., 2005).

Sulfides are proposed to inhibit butyrate oxidation in epithelial cells by inhibiting short chain acyl-CoA dehydrogenation of activated fatty acids which is an important rate limiting step in the oxidation of SCFA (Bremer and Osmundsen, 1984; Moore et al., 1997). Although the inhibitory effect of short chain acyl-CoA dehydrogenase has been well recognized, the mechanism underlying the negative interrelation between sulfides and short chain acyl-CoA dehydrogenase is not fully understood (Babidge et al., 1998). Different mechanisms have been proposed to account for how sulfides can inhibit short chain acyl-CoS. Sulfides may inhibit short chain acyl-CoA by forming bytyrate CoA–persulfides (Shaw and Engel, 1987; Babidge et al., 1998). Babidge et al., (1998) also proposed that sulfides may inhibit short chain acyl-CoA by inactivating the electron transfer flavoprotein which is specific to the activity of short chain acyl – CoA dehydrogenase. Inhibited activity of short chain acyl-CoA dehydrogenase will result in impaired β-oxidation of SCFA, which subsequently causes an energy deficiency state in epithelial cells (Roediger et al., 1993a). In addition, Blachier et al. (2009) proposed that since the inhibitory effect of sulfide on acetate oxidation has been identified, the inhibition of cytochrome c oxidation by sulfide rather than the inhibition of short chain acyl-CoA dehydrogenase might be major factor associated with SCFA oxidation inhibition in epithelial cells.

Inhibitory effect of sulfides on epithelial butyrate metabolism has been considered to have pathogenic implications in Ulcerative Colitis, which is a common bowel disease of humans (Pitcher and Cummings, 1996; Moore et al., 1997; Roediger et al., 1997; Jørgensen and Mortensen, 2001; Leschelle et al., 2005).

Short chain fatty acid oxidation in epithelial cells is important in maintaining healthy

epithelial barrier function. Since SCFA oxidation provides fuels for the absorption of ions, muscle synthesis, lipid synthesis of membrane, and phase II detoxification process in epithelial cells, inhibition of butyrate oxidation will lead to the destruction of the epithelial cell barrier (Kitajima et al., 1999). The impaired epithelial barrier will cause massive transport of some toxic ions, which will pose a threat to the whole-body health. Therefore, SCFA metabolism plays critical roles in maintaining normal absorptive function of epithelial cells.

Relationship between high dietary S and SCFA absorption from the rumen has not been evaluated. Since SCFA metabolism in epithelial cells is inhibited by sulfides, it is possible that the absorption of SCFA would also be influenced by sulfides as absorption of SCFA is influenced by the SCFA metabolism in epithelial cells.

Sulfide is produced by the reduction of inorganic sulfate or sulfite, as well as the degradation of S containing amino acids in the rumen. Sulfide in the rumen is primarily used for microbial S containing amino acid synthesis (Lewis, 1954). Sulfide absorption across the rumen wall is the major route for sulfide disappearance from the rumen (Bray, 1969). Therefore, when more sulfide is absorbed into epithelial cells, SCFA metabolism in epithelial cells would be inhibited, which could subsequently cause a reduced SCFA absorption from the rumen. Therefore, it is interesting to understand the relationship between high dietary S and the SCFA absorption in cattle.

2.7. Sulfur Sources

2.7.1. Sulfur in Feed

The S consumed by animals originates mainly from two sources such as feed and water. Different feedstuffs contain different amounts of S. The variation in S content of feedstuffs may

be due to several factors such as different soil types, different geographical location as well as the inherited characteristics of the plant (Olkowski, 1992). Feedstuffs such as mature forages and corn silage and sorghum forage contain little S (NRC, 1996). However some feedstuffs such as ethanol byproducts and canola meal are rich in S.

2.7.1.1. Sulfur in Dried Distiller's Grains with Solubles

The ethanol industry started to grow in Brazil and U.S in the mid 1970's as it was considered as an alternative to imported crude oil (Gilmour, 1986). Since then the ethanol industry has undergone a rapid expansion due to the widespread demand for environmentally acceptable fuels, as well as its importance on local agricultural economies. The dramatic increase in ethanol production results in increased quantities of by-products available to feed manufactures. The main byproducts after fermentation of starch in ethanol plants are distillers grains (Cezanne et al., 2010). Corn is the most common grain used for ethanol production in the North America owing to its abundance and its greater ethanol yield compared to other cereal grains (Mustafa et al., 2000). Wheat, however, is predominantly used in western Canada due to availability.

The most common ethanol byproduct available for livestock producers is DDGS. Dried distillers grains with solubles has a threefold increase in content of proteins, fat, fiber and some minerals relative to the parent grain due to the removal of starch during the fermentation process (Weigel et al., 1997). Since DDGS is rich in nutrients, it is widely available for livestock feeding as a protein and/or energy source (Weigel et al., 1997). In addition, as the cost of feeding beef cows is rising steadily as a result of increased cost of grain and forage, DDGS is

gaining popularity as a feedstuff for beef cattle because of availability and cost (Leupp et al., 2009).

With the increased use of DDGS in the feedlot rations, considerable research has been conducted to evaluate the effect of corn and wheat DDGS in feedlot cattle rations on cattle performance and carcass quality (Ham et al., 1994; Beliveau and McKinnon, 2008; Buckner et al., 2008). Several research studies have also evaluated the digestibility and fermentation characteristics associated with feeding corn and wheat DDGS as an energy source in corn or wheat-based rations (Leupp et al., 2009; Spiehs and Varel, 2009; Walter et al., 2010). The results of these studies suggest that DDGS can be a good energy and protein source with no adverse effect on the performance and carcass quality of the feedlot cattle.

However, a concern with feeding DDGS is the potential for S-induced PEM due to the relatively high S concentration in DDGS (Buckner et al., 2008). DDGS has almost three fold higher S content than its parental grains, which is mainly due to the process in the ethanol plant (Batal and Dale, 2003). There are two sources of S found in DDGS. One source is the concentration of S in the parental grain used for fermentation. The second source is the S that is added during the fermentation process. The S in the parental grain is organic S mainly cysteine and methionine while the S added during the process is inorganic S. It has been reported that the organic S in corn DDGS was around 0.2% (wt/wt, as received basis). The inorganic S ranged from almost zero up to 0.8% or higher (Zhang, 2010). The S content of DDGS varies based on concentration of S in parental grain and the amount of S applied in the different ethanol plants. The S levels of wheat DDGS from the western Canadian ethanol plants are ranging from 0.4% to

1.1% (Personal communication with Dr. J.J. McKinnon, University of Saskatchewan, 2010). The S levels of corn DDGS are 0.4% -1.3% (Crawford, 2007).

2.7.2. Sulfur in Water

Water is another major S source that contributes to total S intake. Water S content differs among the different geographical locations. Sulfate is the major S form in the water (Olkowski, 2009). The level at which water sulfate can become toxic to cattle is not clearly determined, as it is greatly influenced by the dietary S intake. It is recommended by NRC, (2005) that water for feedlot cattle should contain less than 600 ppm sulfate. In general, water sulfate levels under 1000 pm is considered safe for cattle (Crawford, 2007; Wright, 2007). According to Olkowski (2009), the water sulfate level in some Canadian farms was relatively high and exceeded the safe sulfate level. It was reported that the sulfate concentration in some Saskatchewan farms was three or more times higher than the safe sulfate level (Olkowski, 2009). Thus, these cattle consuming high sulfate containing water are more susceptible to S toxicity. The adverse effects of high water sulfate on animal performance have been studied by several studies (Loneragan et al., 2001; Smiricky-Tjardes et al., 2004). Several cases of PEM associated with high water sulfate have been reported (Gooneratne et al., 1989b; Haydock, 2003; De Sant'Ana and Barros, 2010). Sulfur in water is a major potential source for cattle to increase total S intake. Hence attention should always be given to the water S level in order to avoid excess total S intake.

2.8. Summary

There is no doubt that excess dietary S intake is a major factor causing PEM in cattle. The concern with excess S intake prevents producers from taking the opportunity to overcome

high grain prices by replacing cereal grains with higher levels of DDGS than currently fed. Excess S intake associated PEM has occurred all over the world and caused significant economic loss in the last three decades. However, the role that S plays in producing PEM remains unclear. As discussed, the possible interactions between excess S with other nutrients may have implications in S-induced PEM, which may contribute to the complexity of defining the mechanism of S-induced PEM. Understanding S metabolism and the potential interaction mechanism of high S with other nutrients in the diet is important to determine the mechanism of S-induced PEM.

The hypothesis of the research conducted for the thesis was that high dietary S may influence thiamine metabolism and reduce mineral status, which may subsequently be involved in the development of PEM lesions in cattle. Such adverse effects of high dietary S may be influenced by the nature of the diet. The overall objectives of the studies contained in this thesis were: 1) to evaluate S and thiamine metabolism, mineral status, and SCFA absorption in cattle fed high S containing diet with different F:C ratio, and 2) to evaluate brain thiamine metabolism and mineral status of S-induced PEM affected cattle.

3.0 EFFECTS OF FEEDING DRIED DISTILLERS GRAINS WITH SOLUBLES FROM CORN, WHEAT OR A 50:50 CORN:WHEAT BLEND ON SERUM SULFATE LEVELS OF FEEDLOT STEERS[1]

3.1. Abstract

The effect of feeding corn (CDDGS), wheat (WDDGS) or a 50:50 corn:wheat blend (BDDGS) DDGS on serum sulfate concentration in feedlot steers was evaluated using 288 crossbred steers (273.9 ± 18.5 kg) in a completely randomized design. The steers were backgrounded and finished. The control backgrounding diet consisted of 34.3% rolled barley grain, 26.0% brome grass hay, 10.3% barley straw, 22.8% barley silage, and 6.7% supplement (DM basis). For the three treatment diets, 17% of the barley grain was replaced with DDGS. Sulfur concentrations of control, BDDGS, CDDGS and WDDGS diets in the backgrounding phase were 0.2, 0.23, 0.31 and 0.33% (DM), respectively. The control finishing diet was 86.8% rolled barley grain, 5.8% supplement and 7.4% barley silage (DM), and the three DDGS treatments included replacement of 40% of the barley grain (DM) with an equal amount of DDGS. The corresponding sulfur concentrations for control, BDDGS, CDDGS and WDDGS diets in were 0.2, 0.33, 0.51 and 0.65% (DM), respectively. Cattle fed CDDGS or WDDGS exhibited higher ($P < 0.01$) serum sulfate levels compared to BDDGS or control cattle in both backgrounding and finishing phases. No difference ($P > 0.05$) in serum sulfate concentration was noted between the cattle fed WDDGS and CDDGS. Serum sulfate concentration of BDDGS cattle in the backgrounding phase did not differ ($P > 0.05$) from the control cattle, while it was

[1] A version of this Chapter has been published: Amat, S., Hendrick, S., McAllister, T.A., Block, H.C., and McKinnon, J.J. 2012. Effects of distillers' dried grains with solubles from corn, wheat or 50:50 corn:wheat blend on performance, carcass characteristics and serum sulfate levels of feedlot steers. Can. J. Anim. Sci. 92(3):343-351

higher ($P < 0.01$) than control cattle in the finishing phase. The overall serum sulfate concentration in cattle fed CDDGS and WDDGS treatments was lower ($P < 0.01$) in finishing phase relative to backgrounding phase despite higher S intake during finishing ($P < 0.01$). The results of this study suggest that serum sulfate levels in cattle fed DDGS based backgrounding or finishing diets reflect the dietary S intake. Serum sulfate levels may be influenced by the nature of the diet (i.e. high grain vs. forage).

3.2. Introduction

The Canadian ethanol industry is growing as government policy mandates the renewable source of energy. Canadian ethanol plants differ from those in the United States in that they use both wheat and corn feedstock depending upon the value of each. The dramatic increase in ethanol production has resulted in increased quantities of byproducts such as DDGS that are available for beef producers. Dried distillers grains with solubles has higher protein, fat, fiber and some minerals than the parent grains due to fermentation of starch (Weigel et al., 1997). Both WDDGS and CDDGS have become common energy and protein sources in feedlot rations in western Canada (Yang et al., 2012).

A concern with feeding DDGS is the potential for a high dietary S concentration that can induce PEM in cattle (Gould, 1998; Buckner et al., 2008). Polioencephalomalacia is a neurological disease characterized by focal necrosis of the cerebral cortex (Roberts and Boyd, 1974). Sulfur toxicity has been recognized as the major factor causing PEM (Olkowski, 1997; Gould, 1998). Wheat DDGS contains up to 1.1% S (Personal communication with Dr. J.J, McKinnon, University of Saskatchewan, 2010) which is higher than that of the original wheat

(0.14%) (NRC, 2000). This high S is a result of both concentration of S in the parent grain due to fermentation as well as addition of sulfuric acid to the fermentor to control pH (Zhang, 2010). This latter practice varies between ethanol plants and leads to variation in S content of DDGS from different plant sources.

Outbreaks of PEM associated with feeding CDDGS has been reported (Buckner et al., 2007; Richter et al., 2012); however, the mechanism of S-induced PEM is not fully understood (Olkowski, 1997). The inhalation and absorption of eructed H_2S gas from the rumen is proposed to be a major factor inducing PEM (Gould, 1998). Measuring ruminal H_2S has occasionally been used to identify cattle at risk of PEM. However, the ruminal H_2S concentration in clinically affected animals is often lower than that of other healthy animals in the same herd (Gould, 1998; Niles et al., 2002a). In addition, no conclusive evidence is available to support that the amount of inhaled H_2S gas is sufficient enough to cause malacic lesions in cattle (Olkowski, 1997; Niles et al., 2002a). As such, ruminal H_2S measurement remains a questionable method to assess the risk of PEM. Thus, there is a need to discover an indicator in cattle that allows for the identification for the risk of PEM.

Serum sulfate is one potential indicator. Dietary S is metabolized in the rumen to sulfide (Lewis, 1954). Sulfide is absorbed across the rumen wall and transported to the tissues via the bloodstream (Anderson, 1956). In tissues, sulfide is oxidized to sulfate (Curtis et al., 1972). Since the sulfate is the end product of sulfide metabolism, we hypothesize that measuring serum sulfate may be a means to identify cattle with potential risk of PEM. Few studies have evaluated the relationship between serum sulfate and S intake from the typical diet (Weir and Rendig, 1954)or water (Hansard and Mohammed, 1969). To date, no information is available on serum

sulfate levels in cattle fed DDGS. Therefore, the objective of present study was to evaluate the effects of feeding DDGS from corn, wheat or a 50:50 corn:wheat blend DDGS on serum sulfate levels of feedlot steers.

3.3. Materials and Methods

3.3.1. Animals, Management and Diet Composition

Cattle used in this trial were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993), under the University of Saskatchewan Animal Care Protocol 19910012. In November 2008, 288 crossbred steers with an average weight of 273.9 ± 18.5 kg were purchased and transported to the Beef Cattle Research Centre at the University of Saskatchewan, Saskatoon, SK. The steers were randomized to 24 pens and each pen was randomly assigned to 1of 4dietary treatments. The trial included a 70-d backgrounding period, followed by a finishing period with a target end-point of 643 kg. Initial processing included ear tagging for identification, treatment for parasites (Ivomec®, Merial Canada, Baie D'Urfe', QC), implanting with Synovex S® (Pfizer Canada, Inc., Kirkland, QC), as well as vaccinating for clostridial diseases and *Histophilus somni* (UltraBac® 7/Somnubac®, Pfizer Canada, Inc., Kirkland, QC), infectious bovine rhinotracheitis, parainfluenza 3, bovine respiratory syncytial virus, bovine viral diarrhea (Bovi-Shield® Gold 5, Pfizer Canada, Inc., Kirkland, QC), Pasteurella haemolytica (One Shot® Pfizer Canada, Inc. Kirkland QC), and treatment with a long-acting antimicrobial (Liquamycin® LA, Pfizer Canada, Inc., Kirkland, QC). At the start of the finishing period, cattle were implanted re-implanted with Synovex Plus (Pfizer Canada, Inc., Kirkland, QC).

During the backgrounding period, the control diet consisted of 34.3% rolled barley grain, 26.0% brome grass hay, 10.3% barley straw, 22.8% barley silage, and 6.7% supplement (DM basis). For the three treatments, 17% of the barley grain was replaced with corn, blend or wheat DDGS. At the start of the finishing period, five step-up rations were used to transition the cattle from the backgrounding to the finishing diets by increasing the level of rolled barley and DDGS (~10% DM basis) in the diet and lowering the forage by a corresponding amount every 3 d. The final control diet during finishing averaged 86.8% rolled barley grain, 5.8% supplement and 7.4% barley silage (Table 3.1). The three DDGS treatments included replacement of 40% of the barley grain (DM basis) with an equal amount of CDDGS, BDDGS or WDDGS. Steers were fed the same DDGS sources in the backgrounding and finishing phase. Rations were formulated to meet or exceed NRC (1996) recommendations for CP, minerals and fat-soluble vitamins for each stage of growth. The Ca:P ratio was formulated to range from 1.5:1 to 2:1 with limestone added to the supplement as the DDGS content of the ration increased.

The barley silage (AC Rosser) used in the study was grown at the University of Saskatchewan farm, harvested and stored in a bunker silo. Barley silage samples were taken every 2 wk with the DM content recorded and used to adjust daily feeding amounts as necessary. Barley grain (65.1 ± 0.96 kg/hL, mean ± SD) was purchased from commercial sources and dry rolled on site (Ross Kamp Champion, Waterloo, IA). The WDDGS was supplied by Terra Grain Fuels (Belle Plaine, SK).The CDDGS was purchased in 2loads from ConAgra Foods (Omaha, NE) and from Blue Flint Ethanol, (Underwood, ND). The BDDGS was supplied by Husky Energy Inc. (Lloydminister, SK) and was the by-product of an ethanol fermentation run that used a 50:50 wheat and corn grain mix. In both trials, cattle were fed *ad libitum* with feed delivered

Table 3.1. Ingredient composition and chemical analysis of control, wheat (WDDGS), corn (CDDGS), 50:50 corn:wheat blend (BDDGS) diets

	Backgrounding diet				Finishing diet			
	Control	WDDGS	CDDGS	BDDGS	Control	WDDGS	CDDGS	BDDGS
Diet Composition (%DM Basis)								
Barley silage	22.8	22.4	22.6	22.9	7.4	7.2	7.3	7.2
Brome grass hay	26.0	26.8	26.8	26.2	-	-	-	-
Barley straw	10.3	10.3	10.4	10.3	-	-	-	-
Supplement	6.7	6.7	6.7	6.7	5.8	5.8	5.8	5.8
Barley grain	34.2	16.8	16.6	16.8	86.8	47.1	46.2	46.8
WDDGS	-	17	-	-	-	39.9	-	-
CDDGS	-	-	16.9	-	-	-	40.7	-
BDDGS	-	-	-	17.1	-	-	-	40.2
Supplement composition (% DM basis)								
Barley	37.4	71	71	71	28.7	37.2	37.2	37.2
Canola oil	-	-	-	-	3.2	3.2	3.2	3.2
Canola meal	29.4	-	-	-	21.7	-	-	-
Limestone	3.3	8.9	8.9	8.9	24.1	37.7	37.7	37.7
Ionophore premix[z]	6.2	6.2	6.2	6.2	-	-	-	-
Ionophore/thiamine premix[y]	-	-	-	-	8.7	8.5	8.5	8.5
Urea	9.9	-	-	-	-	-	-	-
Mineral salt[x]	5	5	5	5	4.9	4.9	4.9	4.9
LS 106[w]	8.8	8.9	8.9	8.9	8.7	8.5	8.5	8.5
Chemical analysis (% DM basis ± SE)								
Crude protein	11.68 ± 0.16	14.37 ± 1.32	13.21 ± 0.61	13.15 ± 2.07	11.73 ± 0.72	23.38 ± 1.08	18.29 ± 0.19	19.94 ± 0.45
Ether extract	1.96 ± 0.27	2.89 ± 0.12	4.38 ± 0.25	3.58 ± 0.81	2.57 ± 0.12	4.43 ± 0.08	8.29 ± 0.50	6.48 ± 0.26
Calcium	0.56 ± 0.11	0.67 ± 0.06	0.58 ± 0.18	0.62 ± 0.08	0.55 ± 0.06	0.71 ± 0.17	0.73 ± 0.26	0.83 ± 0.12
Phosphorus	0.30 ± 0.01	0.38 ± 0.04	0.38 ± 0.02	0.38 ± 0.06	0.35 ± 0.02	0.55 ± 0.02	0.47 ± 0.06	0.58 ± 0.07
Sulfur[v]	0.20 ± 0.02	0.33 ± 0.03	0.31 ± 0.03	0.23 ± 0.01	0.2 ± 0.03	0.65 ± 0.06	0.51±0.02	0.33±0.02

[z]Ionophore Premix contains 96.8% barley and 3.2% Rumensin® Premix (as monensin sodium at 200 g kg^{-1} , Elanco, Guelph, ON) (DM basis)

[y]Ionophore/Thiamine Premix contains 94.6 % barley, 3.2% Rumensin® Premix (as monensin sodium at 200 g kg^{-1} , Elanco, Guelph, ON) and 2.2% thiamine premix (137.5 g kg^{-1} thiamine) (DM basis)

[x]Mineral salt: 95 % NaCl, 12 000 ppm Zn, 10 000 ppm Mn, 4000 ppm Cu, 400 ppm I, 60 ppm Co, 30 ppm Se

[w]University of Saskatchewan vitamin A & D supplement= 440,500 IU vitamin A, and 88,000 IU vitamin D_3 kg^{-1}

[v]Sulfur contents of wheat DDGS, corn DDGS, blend of corn and wheat DDGS were 1.1, 0.78 and 0.38 %(DM), respectively

twice daily in two equal allotments at approximately 0900 and 1500 h.

3.3.2. Data Collection and Analysis

The amount of feed delivered to each pen was recorded daily. Every two weeks, the bunks were cleaned and orts weighed. Samples of the total mixed ration were collected twice a month from each pen, while barley, DDGS and supplement samples were taken as each load was received.

Blood samples (two 10-mL vacutainer tubes) were collected from the jugular vein of 96 randomly selected steers (4 steers per pen) immediately prior to start and upon completion of both the backgrounding and finishing phases. Blood samples were transported immediately to the Western College of Veterinary Medicine, Saskatoon, SK, where they were centrifuged for 15 min at 1600 g. Serum was then transferred to cryovials and stored at -80^{o}C for serum sulfate analysis. Water samples (n = 2) from the South Saskatchewan River were collected from a dugout that was used to water the cattle for sulfate analysis.

3.3.3. Chemical Analysis

Sulfur from the feed samples was analyzed using a Leco S-144DR S Combustion Analyzer (Leco Corporation, 3000, Lave view Ave, St. Joseph, MI) with the use of tungsten oxide as a combustion aid by the Cumberland Valley Analytical Services (CVAS, Hagerstown, MD). Serum sulfate was analyzed with high performance ion chromatography (HPIC) according to Russo et al. (1998) at the Veterinary Diagnostic Laboratory of Colorado State University (Fort Collins, CO). Water sulfate was determined using inductivity coupled plasma atomic emission spectroscopy (ICP-AES) according to the standard methods for the examination of water and

waste water by the Saskatchewan Research Council Analytical Laboratory (Saskatoon, SK)

3.3.4. Statistical Analysis

Data were analyzed as a completely randomized design using the Mixed Model Procedure of SAS (Version 9.2; SAS Institute, Inc. Cary, N. C.). Pen was used as the experimental unit. The Kenward-Roger adjustment was used to determine degrees of freedom. Means were compared by protected least significant difference with an alpha of 0.05. The mean serum sulfate concentration or the mean S intake from the cattle fed WDDGS and CDDGS were obtained. These means were compared between backgounding and finishing phases as Two Sample T- test using SAS TTEST Procedure.

3.4. Results and Discussion

Sulfur intake was consistently higher (P = 0.01) on the CDDGS and WDDGS diets throughout the trial (Table 3.2). This reflected the S content of the 3 DDGS sources. Both CDDGS and WDDGS were markedly higher in S content (average 0.90%) than the BDDGS (0.40%). All 3 DDGS types originated from different plants. It is likely that differences in S levels reflect management practices of the plant and are not reflective of differences between cereal gain types or mixtures. No attempt was made to estimate sulfate intake from water as consumption was not available. However, water quality as indicated by sulfate levels was very good, averaging 57.5 ± 0.71 mg/L (Olkowski, 2009).

Table 3.2. Comparison of dietary sulfur intake in cattle fed wheat (WDDGS), corn (CDDGS) or a 50:50 corn:wheat blend (BDDGS) dried distillers grains with solubles (DDGS) among different feeding stages

	Dietary treatment					
	Control	WDDGS	CDDGS	BDDGS	SEM[z]	*P*-value
Backgrounding phase (g/d)	16.71[b]	26.03[a]	23.33[a]	17.50[b]	0.81	<0.01
Finishing phase (g/d)	20.94[d]	72.26[a]	55.35[b]	36.81[c]	0.654	<0.01

[z]SEM = pooled standard error of the mean
[a-d]Means within raw with different superscripts are significantly different ($P < 0.05$)

Sulfur intake influenced serum sulfate levels in both the backgrounding and finishing phases. In the backgrounding phase, cattle fed WDDGS (179.8 ± 11.3 ppm) and CDDGS (178.0 ± 17.1 ppm) had higher ($P < 0.01$) serum sulfate levels than those fed the control (123.3 ± 7.7 ppm) or BDDGS (119.5 ± 18.5 ppm) diets. But no difference in serum sulfate concentration was found between WDDGS and CDDGS ($P > 0.05$) (Figure 3.1). High serum sulfate concentration of the cattle fed wheat and corn DDGS in backgrounding phase reflected the S intake and S level of the wheat and corn DDGS (Figure 3.1; Table 3.2). Cattle fed the BDDGS diet however, showed similar ($P > 0.05$) serum sulfate levels as those fed the control diet. Presumably the lower S in BDDGS ration resulted in the low serum sulfate concentration. Similar responses ($P < 0.01$) were noted at the end of the finishing period (Figure 3.1) with an exception of the cattle fed BDDGS diet exhibited higher ($P > 0.01$) serum sulfate than those fed the control diet. These results indicated that serum sulfate reflects differences in dietary S intake. Our results are in agreement with Weir and Rendig (1954), who observed that the serum sulfate was a positive reflection of total S intake in sheep. It has also been reported that serum sulfate increased with higher S intake from water (Hansard and Mohammed, 1969).

The maximum tolerable S concentration in the diet for cattle is 0.4% (DM base) (NRC, 2000). The S concentrations in WDDGS and CDDGS finishing diets (0.51% and 0.65%, respectively) exceeded this maximum tolerable level (Table 3.1). While the S concentration in BDDGS finishing diet was under the tolerable level (0.33% S). In addition, the cattle fed WDDGS and CDDGS showed similar pattern in that serum sulfate concentration was higher than the control in both backgrounding and finishing phases. Therefore, the overall serum sulfate concentration in cattle fed those two DDGS treatments were used to evaluate the difference in serum sulfate concentration between the two feeding phases. Although dietary S intake for those cattle fed CDDGS and WDDGS in the backgrounding phase was lower ($P < 0.01$) (Table 3.3), serum sulfate levels were higher ($P < 0.01$) during backgrounding than finishing phase (179.8 ± 13.94 vs. 153.8 ± 18.27 ppm).

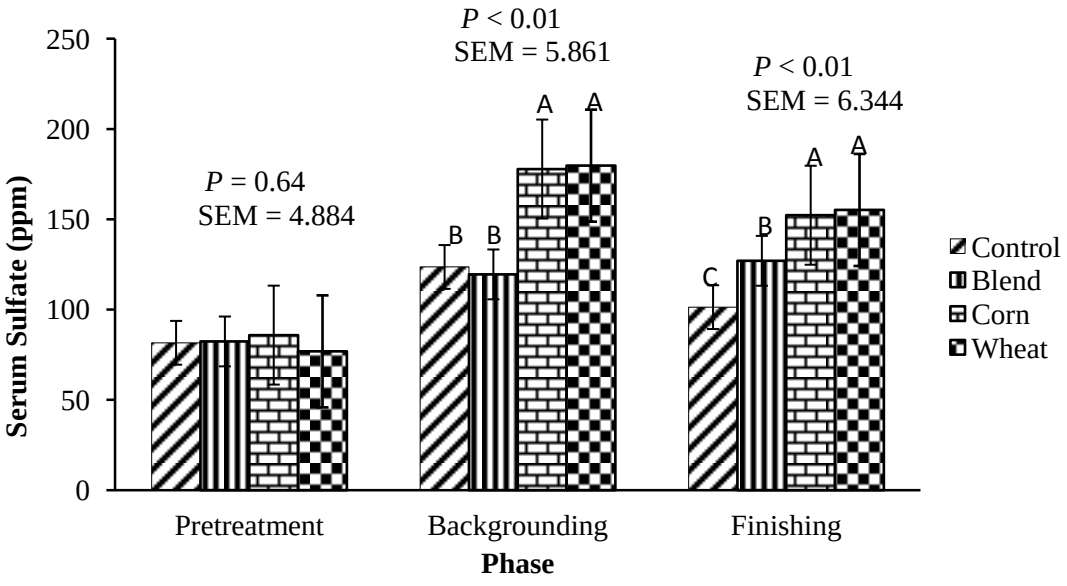


Figure 3.1. The effect of feeding wheat (WDDGS), corn (CDDGS) or a 50:50 corn:wheat blend (BDDGS) dried distillers grains with soluble (DDGS) on serum sulfate concentration of steers. Means with different letters are significantly different ($P < 0.05$).
SEM = pooled standard error of the mean

Table 3.3. Overall comparison of serum sulfate concentration or dietary sulfur intake of cattle fed wheat (WDDGS) and corn (CDDGS) dried distillers grains with soluble (DDGS) between backgrounding and finishing phases

	Phase		
	Backgrounding	Finishing	*P*- value
S intake	25.0 ± 1.60	63.8 ± 9.05	< 0.01
Serum sulfate	179.8 ± 13.94	153.8 ± 18.27	< 0.01

[z]SEM = pooled standard error of the mean

It is possible that the high grain in the finishing diet may have led to the reduced serum sulfate concentration in finishing steers. However, the mechanism by which a high grain diet affects S metabolism needs to be investigated. Neville et al. (2010) suggested that there are interactive effects of dietary S, thiamine supplementation, and dietary grain supplementation in finishing rations. In this research, lambs receiving finishing diets containing 60% DDGS with either 0.73% or 0.87% S did not develop any clinical PEM lesions. The results of the present study suggests that there may be an interaction between the grain content of the diet and dietary S intake on serum sulfate concentration. Cattle fed high grain diet in the finishing phase showed lower serum sulfate concentration relative to those fed high forage based backgrounding diet despite higher S intake.

One of the possible reasons why serum sulfate may be influenced by the grain level of the diet is that the rumen sulfide absorptive capacity may be impacted by grain feeding. Absorption across the rumen wall is the main route for sulfide disappearance from the rumen (Anderson, 1956; Bray, 1969). It is possible that when cattle are fed high grain diets, the epithelial barrier function is enhanced (Lodemann and Martens, 2006) and this may prevent sulfide translocation across the rumen wall thereby reducing the concentration of serum sulfate. Further work is required to determine the absorption of dietary S in cattle fed high forage backgrounding and

high grain finishing rations.

No clinical signs of PEM were observed during the backgrounding or finishing phases. Thiamine was supplemented at 15 mg/kg (DM basis) during the finishing phase of the trial (Table 3.1). Four animals either were removed or died during the study due to reasons unrelated to dietary treatment. As suggested by Neville et al. (2010), the relationship between dietary S level and incidence of PEM in ruminants is not clear.

3.5. Conclusion

The results showed that serum sulfate levels in feedlot steers fed different sources of DDGS varies with the S content of the DDGS. Wheat DDGS and CDDGS resulted in highest serum sulfate concentration in both backgrounding and finishing phases. In addition, the overall comparison in serum sulfate concentration of steers fed WDDGS and CDDGS between backgrounding and finishing phases indicates that high grain diets may affect the serum sulfate concentration. Therefore, further research is needed to evaluate the effect of high grain diets on serum sulfate and on S-induced PEM.

4.0 EFFECTS OF DIETARY SULFUR CONCENTRATION AND FORAGE-TO-CONCENTRATE RATIO ON RUMINAL FERMENTATION, SULFUR METABOLISM, AND SHORT CHAIN FATTY ACID ABSORPTION IN BEEF HEIFERS

4.1. Abstract

This study was conducted to evaluate the effects of dietary S concentration and F:C on ruminal fermentation, S metabolism and SCFA absorption in beef heifers. Sixteen ruminally cannulated heifers (initial BW 628 ± 48 kg) were used in a randomized complete block design with a 2 × 2 factorial treatment arrangement. Main factors included the F:C ratio (4% forage vs. 51% forage, DM basis), and the S concentration which was modified using differing sources of wheat DDGS to achieve low and high S diets (LS = 0.30% vs. HS = 0.67% S DM basis, respectively). Elemental S was also added to increase the S content of the HS diets. Blood, rumen fluid, rumen gas cap and urine samples were collected for serum sulfate, ruminal S^{2-}, H_2S, and urinary sulfate analysis, respectively. Continuous rumen pH and SCFA (acetate, butyrate and propionate) absorption were measured. There was no interaction between S concentration and the F:C. The F:C did not ($P = 0.26$) affect DMI or S metabolism ($P > 0.05$) but ruminal pH was reduced ($P < 0.01$) and SCFA absorption was greater ($P < 0.01$) for low F:C diets. Heifers fed HS diets had reduced DMI ($P < 0.01$) and greater rumen pH ($P < 0.01$), greater concentrations of ruminal H_2S ($P < 0.01$), serum sulfate ($P < 0.01$) and urinary sulfate ($P < 0.01$), as well as greater urinary sulfate output ($P < 0.01$) relative to LS diets. Ruminal H_2S was positively correlated with serum sulfate ($r = 0.89$; $P < 0.01$). Both HS ($P = 0.06$) and low F:C ($P = 0.07$) diets tended to reduce urine output. Feeding HS diets reduced SCFA absorption ($P < 0.05$). In

summary, S metabolism in beef heifers was not influenced by different F:C, but HS reduced DMI, inhibited SCFA absorption and increased urinary S excretion. Low F:C diet stimulated SCFA absorption.

4.2. Introduction

Excessive dietary S is a known cause of PEM in cattle (Gooneratne et al., 1989a; Gooneratne et al., 1989b; Gould et al., 1991; Gould, 1998; Buckner et al., 2007). Dietary S concentration often exceeds the recommended maximum level (0.4% DM basis) when diets include high S containing feedstock such as DDGS (NRC, 2000). Indeed, outbreaks of PEM have been reported in recent years and are thought to be associated with feeding DDGS (Buckner et al., 2007; Richter et al., 2012). Concerns with the high S content of DDGS can therefore limit its inclusion rate in diets despite its availability in the market and nutritional profile (Leupp et al., 2009; Neville et al., 2010).

Feeding strategies that mitigate S toxicity could be a solution to help prevent PEM, thereby avoiding limitations to ingredient inclusion based on dietary S. It has been suggested that S toxicity may be influenced by diet composition (Vanness et al., 2009; Neville et al., 2010) with greater risk for PEM when feeding high levels of S in high-concentrate diets (Kung et al., 1998). Acidic rumen conditions favour the production of H_2S in the rumen and increase its concentration in the rumen gas cap (Gould, 1998). Maintaining an elevated rumen pH by increasing the proportion of forage is considered an effective way to reduce the incidence of PEM (Vanness et al., 2009). However, recent research has indicated that high concentrate diets may mitigate the effect of dietary S. Neville et al. (2010) reported that lambs receiving a

finishing diet with 60% corn DDGS and either 0.73 or 0.87% S (DM) did not exhibit any clinical signs of PEM. They suggested that this was likely due to the interactive effects of dietary S, dietary grain supplementation, and thiamine supplementation in finishing rations. In addition, a previous study by Amat et al. (2012) indicated that serum sulfate concentration in feedlot steers fed a backgrounding ration containing 0.32% S (DM basis) was higher than when fed a finishing ration which contained 0.58% S (DM basis). It is important to note that in both cases, the source of S was wheat or corn DDGS. Collectively, these findings led to the hypothesis that grain levels in the diet may influence S metabolism and thereby mitigate the toxicity of S.

In addition to inducing PEM, an inhibitory effect of S on acetate and butyrate oxidation in colonocytes has been documented (Roediger et al., 1993b; Roediger et al., 1997; Leschelle et al., 2005; Blachier et al., 2009). However, the relationship between high dietary S and SCFA absorption from the rumen has not been studied. Since SCFA metabolism is involved in regulating concentration gradients between the rumen fluid and cytosol to promote absorption (Gäbel et al., 2001), a reduction in epithelial SCFA metabolism, induced by high S, may decrease ruminal SCFA absorption. The objective of this study was to evaluate the effects of high dietary S concentration and F:C on ruminal fermentation, S metabolism, and SCFA absorption in beef heifers.

4.3. Materials and Methods

Cattle in this experiment were cared for in accordance to the guidelines of the Canadian Council on Animal Care (1993), under the University of Saskatchewan Animal Care Protocol 20100018.

4.3.1. Animals, Experimental Design and Treatments

Sixteen mixed breed and ovariectomized heifers with rumen cannulas (initial BW 628 ± 48 kg) were housed in individual pens (9 m^2) equipped with a feed bunk and water bowl, and rubber floor mats at the University of Saskatchewan Livestock Research Barn. Pens were scraped and cleaned every day before morning feeding. The experiment was conducted as a randomized complete block using a 2 × 2 factorial treatment arrangement with the main effects of dietary S content and F:C ratio. Heifers were grouped by initial BW into 4 blocks: B1 (564 ± 11 kg), B2 (612 ± 23 kg), B3 (652 ± 6 kg) and B4 (683 ± 19 kg). Two heifers per diet type (high F:C vs. low F:C) within each block were randomly assigned to 1 of 4 pens. Pens within block were randomly assigned to 1 of 2 treatments (0.30% vs. 0.67% S on a DM basis) (Table 4.1). The F:C ratio was modified by altering the proportion of barley silage (4% vs. 51% DM basis), whereas, the S content was modified using two different levels of S (0.55% vs. 1.07%, DM) containing wheat DDGS (approximately 38% DM basis) to achieve low and high S diets (0.3% vs. 0.67% S on DM basis, respectively). In order to achieve high S diets with 0.67% S (DM), elemental S (Cat#13803, Sigma-Aldrich, ON, Canada,) was added to the HS supplements.

Heifers were fed their individual diets at 0800 and 1600 h. Diets were formulated to meet the NRC (2000) nutrient requirements of growing beef cattle. The trial included a 31-d adaptation period and a 37-d experimental period. During the adaptation period, a 7-step adaptation was used to transition each heifer on the low F:C treatments; while a 4-step adaptation was used for heifers fed the high F:C treatments. During this adaptation period, low S containing DDGS and pellets containing no elemental S were fed. There were 4 d between each step and 7 d between the final step and the trial diets. Orts were weighed and recorded daily before the

Table 4. 1. Ingredient composition and chemical analysis of treatment diets[z]

	HGHS	HGLS	LGHS	LGLS
Diet composition (%DM Basis)				
Barley silage	3.69	3.71	50.64	50.96
Barley grain	52.97	53.29	-	-
DDGS-TG[y]	37.79	-	39.5	-
DDGS-NWT[x]	-	37.44	-	39.13
Supplement-1	5.56	-	-	-
Supplement-2	-	5.57	-	-
Supplement-3	-	-	9.86	-
Supplement-4			-	9.91
Supplement composition (% DM basis)				
Barley	34.42	36.7	57.7	58.65
Canola oil	3.2	3.21	3.85	3.86
Limestone	35.12	35.23	15	15.02
Ionophore/thiamine Premix[w]	8.54	8.57	12.82	12.84
Trace mineral salt[v]	4.85	4.86	2.78	2.78
LS106[u]	11.39	11.42	6.85	6.86
Elemental sulfur	2.48	-	1	-
Chemical analysis (% DM basis ± SD)				
Crude protein	20.76 ± 0.35	20.70 ± 0.19	21.76 ± 0.35	21.78 ± 0.29
Starch	34.05 ± 0.85	34.13 ± 0.73	16.43 ± 1.17	17.03 ± 0.85
Acid detergent fiber	9.24 ± 0.70	9.35 ± 0.51	20.36 ± 1.15	20.55 ± 0.20
Neutral detergent fiber	25.57 ± 0.54	27.39 ± 0.65	36.47 ± 0.37	38.40 ± 1.25
Calcium	0.67 ± 0.17	0.76 ± 0.02	1.14 ± 0.26	0.96 ± 0.04
Phosphorus	0.56 ± 0.03	0.56 ± 0.00	0.57 ± 0.04	0.57 ± 0.02
Sodium	0.27 ± 0.05	0.26 ± 0.00	0.44 ± 0.07	0.41 ± 0.01
Potassium	0.87 ± 0.01	0.87 ± 0.00	1.59 ± 0.05	1.59 ± 0.05
Sulfur[t]	0.59 ± 0.05	0.29 ± 0.04	0.64 ± 0.03	0.33 ± 0.02

[z]Treatment diets:HGHS: low F:C high S; HGLS: low F:C low S; LGHS: high F:C high S; LGLS: high F:C low S

[y]DDGS-TG: high S containing DDGS (1.07% S DM basis)

[x]DDGS-NWT: low S containing DDGS (0.55% S DM basis)

[w]Ionophore Premix of supplement1 and 2 contains 95% barley, 3% Rumensin® Premix (as monensin sodium at 200 g/kg , Elanco, Guelph, ON) and 2% thiamine premix (DM basis); Ionophore Premix of supplement 3 and 4 contains 98% barley, 1% Rumensin® Premix and 1% thiamine premix (DM basis).

[v]Trace mineral salt: 95 % NaCl, 12 000 ppm Zn, 10 000 ppm Mn, 4000 ppm Cu, 400 ppm I, 60 ppm Co, 30 ppm Se

[u]University of Saskatchewan vitamin A & D supplement= 440,500 IU vitamin A, and 88,000 IU vitamin D_3/ kg

[t]Sulfur contents of barley silage and barley grain were 0.23 and 0.13% (DM), respectively. Sulfur content of supplement 1, 2, 3, and 4 were 1.93, 0.10, 1.03 and 0.11 % (DM basis), respectively.

morning feeding.

Barley silage (AC Rosser) used in the study was grown at the University of Saskatchewan farm, harvested and stored in a bunker silo. Barley grain (60.5 kg/hL) was purchased from commercial sources and dry rolled (Ross Kamp Champion, Waterloo, IA). High S (1.07% S) containing WDDGS was supplied by Terra Grain Fuels (Belle Plaine, SK), while low S (0.55% S) containing WDDGS was purchased from North West Terminal Ltd (Unity, SK). Thiamine was added to all diets at 20 mg/kg (DM) in each of the supplements.

Animals were examined twice daily for any signs of abnormalities suggestive of neurological disease. The signs examined are as following: head pressing against fence, hypersensitive to the sound or touch, teeth grinding, staggering and blindness. One LGHS heifer from block 1 had a bladder infection not related to dietary treatment after 2 wk of the experiment. This heifer was treated and recovered, however no urine sampling was performed on her and no blood, rumen or rumen gas cap samples were collected on sampling d 21.

4.3.2. Sampling and Analysis

Sampling regime: Blood, rumen fluid, rumen gas cap and urine samples were collected on d 1, 7, 10, 14, 21, 28 and 35 of the experimental period (Figure 4.1). Blood and rumen samples were collected simultaneously at 0600, 1200, 1800 and 2400 h, rumen gas cap samples were collected at 1200 h, and urine samples were collected over a 24-h period. A staggered sampling approach was used such that sampling from blocks 2, 3 and 4 were started 2, 10, and 12 d after the first block, respectively.

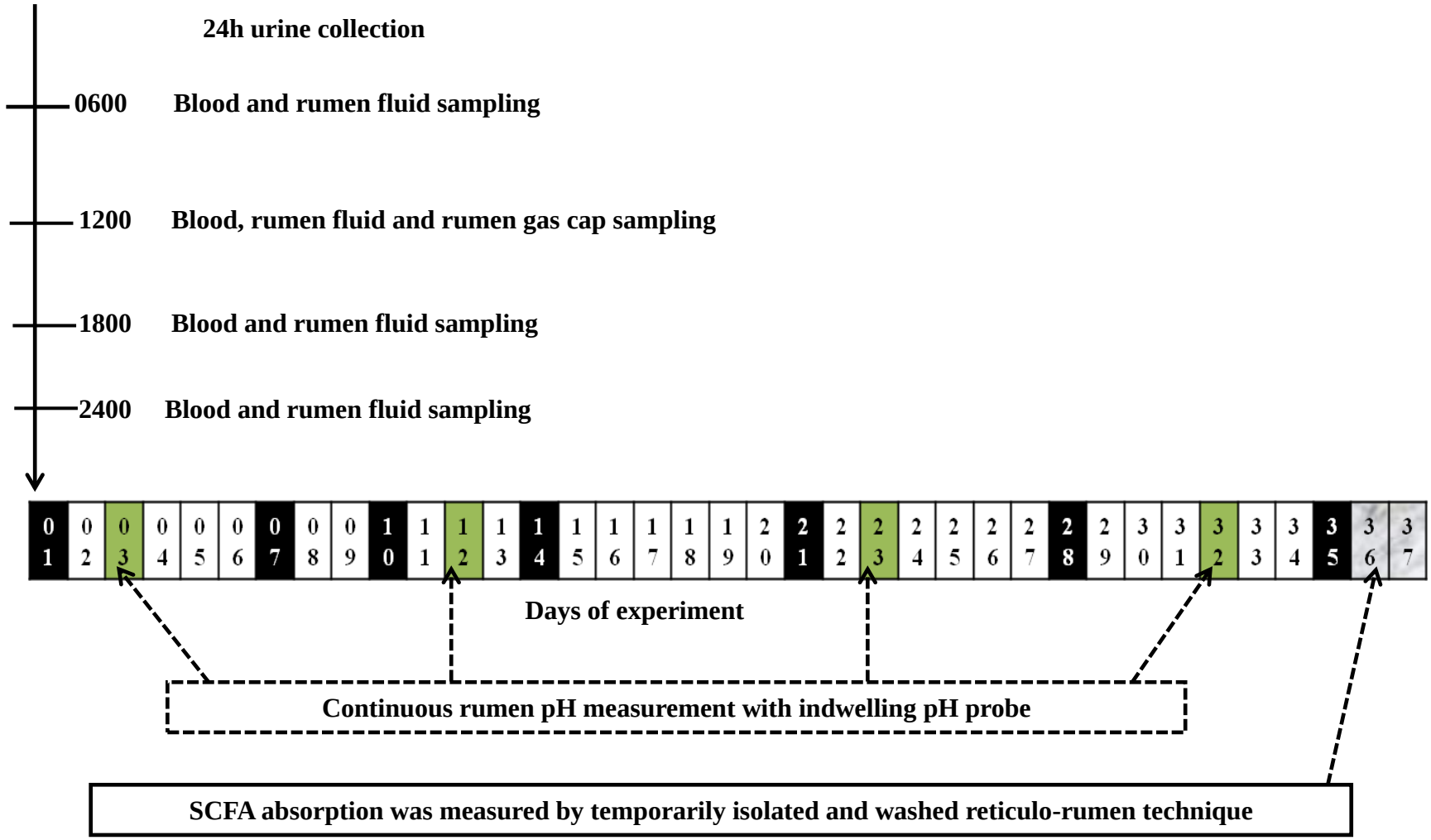
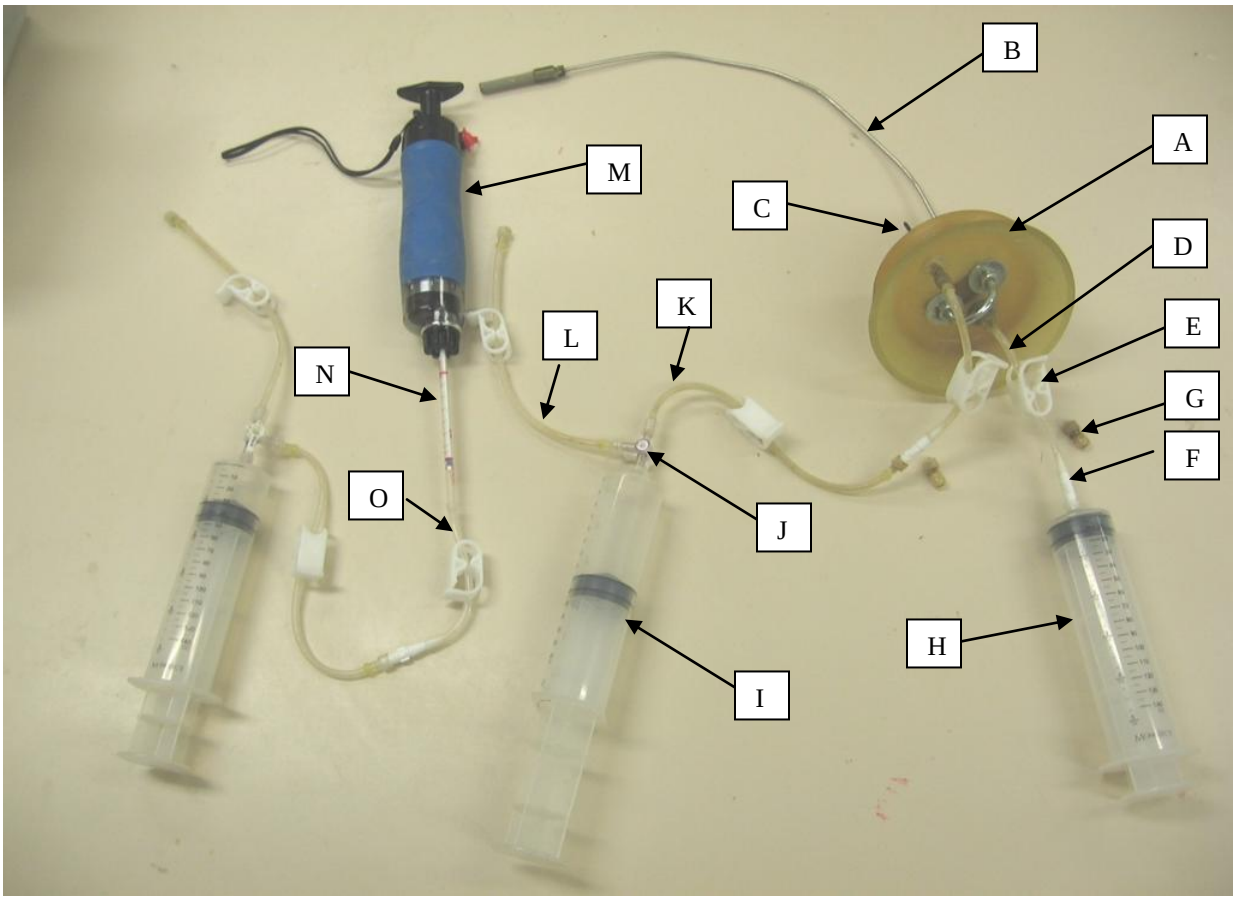


Figure 4.1. Sampling regime

Feed and water sampling and analysis: Feed samples were taken for each batch of barley, supplement pellets and wheat DDGS. Silage samples were taken every week to determine DM content and the diets adjusted accordingly. All feed samples were dried in a forced air oven at 55^{o}C for 72 h and then ground through a hammer mill fitted with a 1 mm screen (Restch ZM 100 grinder, Haan, Germany). Feed samples were analyzed for CP, starch, ADF, NDF, Ca, P, Na, K and S by Cumberland Valley Analytical Services (CVAS, Hagerstown, MD). Water samples from water bowls were collected weekly, composited by equal volume (20 ml) and stored at -20^{o}C for sulfate testing. Water sulfate was determined using ICP-AES by the Saskatchewan Research Council Analytical Laboratory (Saskatoon, SK).

Rumen gas cap and rumen fluid sampling and analysis: In order to keep the integrity of the rumen gas cap, a cannula plug was modified to allow simultaneous rumen gas cap and rumen fluid sampling from cannulated cattle without opening the cannula (Figure 4.2). This modified plug was placed in the animal's cannula 24 h before rumen gas cap sampling. At 1200 h, rumen gas cap was sampled prior to sampling the rumen fluid. Rumen gas cap sampling and measurements were adapted from Neville et al. (2010) with the following modifications. Two 120 mL rumen gas cap samples were drawn into 140 mL syringes. Ruminal H_2S concentration was measured via precision H_2S gas detector tubes attached to a calibrated gas detection pump (Model AP-20S, Sensidyne, Clearwater, FL). Two types of H_2S detector tubes (Sensidyne® No. 120SM and 120SF) were used for the samples collected from the HS cattle and LS cattle, respectively. The concentration of H_2S was recorded from the detector tube by the same individual for each sampling time-point. Duplicate measurements were taken for each heifer and the average of the 2 samples were used for data analysis.

Figure 4.2. Rumen fluid and rumen gas cap sampling apparatus



A,cannula plug (#1EZ Easy-out Stopper, Bar Diamond Inc.);**B**, rumen fluid collection tube (Bar Diamond Ref # 33055, DESC-1); **C**, 4 inch 14 gauge needle; **D**, 8cm-long (4.8 mm diameter) tubing; **E**, plastic tubing clamp; **F**, female luer lock catheter end; **G**, intermittent infusion plug (Argyle, ref # 115006); **H**, 140-ml syringe used for rumen fluid collection; **I**, 140-ml syringe used for rumen gas cap collection; **J**, 3-way stopcock (Smith Medical, ref # MX5311l); K, 15-cm long (4.8 mm diam) tubing used foe sucking the gas from the rumen; **L**, 15cm - long (4.8 mm diam) tubing used for sucking the gas from the syringe to the pump; **M**, gas detection pump (Model AP-20S, Sensidyne®); **N**, precision hydrogen sulfide gas detector tube (Sensidyne® No. 120SM or 120SF); **O**, 6cm-long (4.8 mm diam) tubing with female luer lock catheter end used for connecting detector tube with the tubing of syringe.

Following rumen gas cap sampling, rumen fluid was sampled using a 140 mL syringe.The first 10-15 mL of rumen fluid was discarded and then 100 mL of rumen fluid was collected and transferred to a 250 mL plastic beaker. A 30 mL rumen fluid sample was taken for the measurement of rumen S^{2-}. Ruminal S^{2-} measurement was performed according to Khan et al., (1980) with the following modifications. An Orion DUAL STAR pH/ISE meter (Thermo Scientific, cat# 100-240V) to analyze the ruminal S^{2-}. Ten mL of rumen fluid was placed into a 50 mL beaker and mixed with an equal amount of sulfide antioxidant buffer (SAOB). The SAOB buffer was prepared by dissolving 198.7 m*M* ascorbic acid and 180 m*M* Na_2EDTA in 1 L of 2 *M* NaOH. After the stabilization of S^{2-} ion by the SOAB, the S^{2-} ion was measured with a sulfide ion selective electrode (Orion ion silver/sulfide electrode BN, Thermo Scientific, cat# 9616 BNWP).

Blood sampling and analysis: Blood samples were collected from the jugular vein of all heifers via catheters that were inserted 1 d prior to sampling. Each catheter was flushed with 10 mL of heparinized saline. Ten mL of blood was collected with blood collection tubes containing no anticoagulant (BD Vacutainer, Ref 367820) and centrifuged (15 minutes at 2526 g). Serum was transferred to 4-mL cryovials and stored at -80ºC for sulfate analysis. Serum samples at 1200 and 2400 h sampling points were analyzed for sulfate with HPIC according to Russo and Karmarker (1998) at the Veterinary Diagnostic Laboratory of Colorado State University (Fort Collins, CO).

Urine sampling and analysis: Total urine was collected each sampling day for determination of urine output and sulfate concentration. Urine was collected with bladder catheters (Bardex Foley Catheter, 75 mL capacity balloon; C. R. Bard Inc., Covington, GA.). Bladder catheters were inserted 24 h before the first urinary total collection and remained in

place throughout the trial. Heifers that lost their catheters were recatheterized. During collection, the heifers were haltered with enough space for their normal activities. Total urine was collected into 25 L Nalgene jugs with sterile Nalgene tubing with the weight recorded. Urine was sub-sampled and transferred to 15 mL centrifuge tubes and frozen at –20 ºC for sulfate analysis. Urine sulfate was analyzed with HPIC according to Magee et al. (2004) at the Veterinary Diagnostic Laboratory of Colorado State University (Fort Collins, CO).

In-dwelling rumen pH measurement: On d 3, 12, 23 and 32, heifers were fitted with indwelling rumen pH probes and data loggers (Dascor, Escondido, CA) as described by Penner et al. (2006). The in-dwelling pH measurements allowed for the continuous monitoring (1 min interval) of rumen pH in the ventral sac for 24 h periods. After 24 h, the rumen pH probes were removed from the rumen, the data recorded and the probe recalibrated to pH 4 and 7.

The mV data was combined with the calibration data to calculate pH based on the slope and y-intercept values. Ruminal pH data were usedto summarize minimum, mean and maximum pH. The duration (min/d) and pH area (min/d × pH) under the curve were also calculated using a threshold of pH < 5.5 to summarize severity of ruminal acidosis.

4.3.3. Evaluation of Short Chain Fatty Acid Absorption

The temporarily isolated and washed reticulo-rumen (**WRR**) technique was performed on all heifers to measure SCFA absorption across the reticulo-rumen epithelium. The WRR procedures were performed on 2 heifers from each block at 1300 h on d 36 and the remaining 2 heifers from the same block were subjected to the WRR at 1300 h on the following day (d 37). The WRR technique was described previously by Care et al. (1984). Briefly, reticulo-rumen digesta was completely removed from the rumen and stored in an insulated covered container

until the end of experiment when the digesta was returned to the reticulo-rumen. The reticulo-rumen was washed twice using 10 L of pre-heated tap water (38 ºC). After washing, the excess water was removed using a wet/dry vacuum. Then, 20 L of pre-heated (38 ºC) washing buffer (Table 4.2) was used to wash (5-6 L/wash) the rumen. Washing buffer was poured into the rumen and agitated manually to dislodge material from the rumen epithelium. After each wash, the buffer was removed using the wet/dry vacuum and the procedure was repeated until the effluent was free of digesta. After the reticulo-rumen was completely washed, the reticulo-rumen was isolated from the rest of the gastrointestinal tract. The oesophagus was temporarily occluded using a saliva collection device (University of Leipzig, Leipzig, Germany). The saliva collection device was connected to the vacuum pump (model N86KT45P, KNF Neuberger Inc., Trenton, NJ) and thus allowed the continuous removal of saliva. A Foley catheter (75 mL volume) was used to occlude the omasal orifice. After the occluding the oesophagus and omasal orifice, the reticulo-rumen was washed again with washing buffer (5 L). Fifteen litter experimental buffer with a fluid marker 2 m*M* Co-EDTA (Table 4.2), warmed to 38ºC and adjusted to pH 6.2, was poured into the washed rumen and continually gassed with CO_2.

Sample collection: Samples were collected at 0 (prior to infusion), 5 and 45 min after the experimental buffer was introduced into the reticulo-rumen. Two buffer samples (15 mL and 30 mL) were collected at each sampling time and immediately stored at -20^{o}C for SCFA and Co analysis, respectively. Short chain fatty acid concentration was analyzed using gas chromatography by the Ruminant Nutrition Laboratory of Agriculture and Agri-Food Canada (Lethbridge, AB). Cobalt analysis was analyzed using ICP-MS Spectrophotometer (Thermo Jarrel Ash-Corporation, Franklin, MA) by the Toxicology Laboratory of Prairie Diagnostic Services Inc (Saskatoon, Canada). The osmolality of the experimental buffer (prior to infusion)

Table 4.2. Composition of solutions*

	Composition of washing buffer (mmol/L)	Composition of experimental buffer (mmol/L)
NaCl	105	5
KCl	-	5
$CaCl_2 * 2H_2O$	-	2
$MgCl_2$	-	2
Na-acetate	-	30
K-acetate	-	35
Na-Propionate	20	35
Na-Butryate	-	8
Butyric acid	-	7
$NaHCO_3$	25	25
L-Lactic acid	-	5
Co-EDTA	-	2
$Na_2S *9 H_2O_2$	-	0.2
Acetic acid	10	-
Osmolality	310	314
Actual osmolality		279 ± 9.7

* pH of the buffer was adjusted to 6.2 with NaOH or HCl as needed

was measured in duplicate using advanced instruments osmometer (Model 3250, Advanced Instruments Inc. Norwood, MA).

Calculations for absolute and fractional absorption of SCFA: Buffer preparation accuracy was checked by the concentrations of Co and VFA at 0 sampling point. The initial and final volumes were calculated by the concentrations of Co at the 5 and 45 min sampling points, respectively, using the following formula:

$$C_1V_1 = C_2V_2$$

where C_1 = initial concentration of Co
V_1 = initial volume of buffer in the rumen
C_2 = final concentration of Co
V_2 = final volume of buffer in the rumen

Absolute disappearance of SCFA (mmol/h) was calculated using the formula below:

$$C_{5min}V_{5min} - C_{45min}V_{45min}$$

where C_{5min} = SCFA concentration of buffer in the rumen at time = 5 min
V_{5min} = Volume of buffer in the rumen at time = 5 min
C_{45min} = SCFA concentration of buffer in the rumen at time = 45 min
V_{45min} = Volume of buffer in the rumen at time = 45 min

Fractional disappearance of SCFA (%/h) was calculated using the following formula:

$$[((C_{5min} - C_{45min})/C_{5min}) \times 100]/T$$

where C_{5min} = SCFA concentration of buffer in the rumen at time = 5 min
C_{45min} = SCFA concentration of buffer in the rumen at time = 45 min
T = time = 45 min – 5min = 40 min = 0.67 h

4.3.4. Statistical Analysis

All data, except that of SCFA, were analyzed as a randomised complete block design (RCBD) with repeated measurements with a 2 × 2 factorial treatment arrangement using the Mixed Model Procedure of SAS (Version 9.2; SAS Institute Inc. Cary, NC). Since no significant block effect was observed, the block was removed from the model and data reanalyzed with the

following model: fixed effects of F:C, S, F:C × S, day, F:C × day, S × day and F:C × S × day, and random effect of animal nested within the block. The covariance error structure for each model was chosen based on its lowest AIC value. Short chain fatty acid data were first analyzed as RCB design with 2 × 2 factorial treatments. After observing no significant block effect, the block was removed from the model. The model used for SCFA analysis included fixed effects of F:C, S, and F:C × S, and random effect of animal nested within the block. Correlations between ruminal H_2S and serum sulfate were evaluated using the Proc Reg Procedure of SAS. Satterthwaite method was applied to determine degrees of freedom. Means were compared using Tukey's multiple comparison. Results were considered significant when $P < 0.05$ and trends were discussed when $0.05 \leq P \leq 0.10$.

4.4. Results and Discussion

4.4.1. Sulfur Intake

Actual S concentration in LS and HS diets averaged 0.31% and 0.62%, respectively (Table 4.1). The actual mean S concentration in HS diets was 0.05% lower than the formulated value (0.67%). This discrepancy was a result of slightly lower S content of the supplements to which elemental S was added during pelleting. Average of 0.12 % elemental S was added to the supplements of HS diets. Daily S intake was greater ($P < 0.01$) for heifers fed HS diets relative to those fed LS diets (53.0 vs. 34.5 g/d, respectively) (Figure. 4.3). As expected, no difference ($P = 0.53$) in daily S intake was observed between heifers fed high F:C and low F:C diets (44.4 and 43.0 g/d, respectively). Water S intake was not estimated. However sulfate content of the water used in this trial (110 mg/L) was low (Olkowski, 2009).

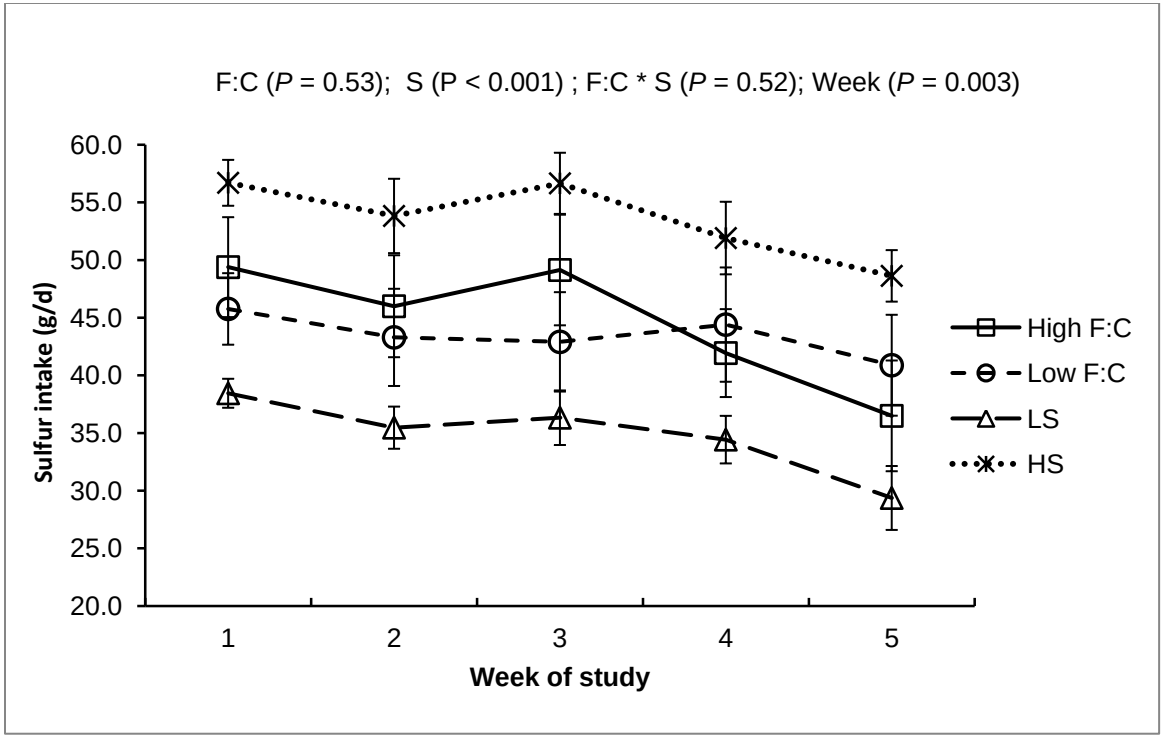


Figure 4.3. The effect of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on sulfur intake of beef heifers

4.4.2. Dry Matter Intake

No interaction was observed between dietary S and F:C for DMI ($P = 0.87$) (Figure. 4.4). Dry matter intake was reduced by 22.5% with high dietary S ($P < 0.001$) but was not affected by F:C ($P = 0.26$). Uwituze et al. (2011a) observed reduced DMI in steers fed corn DDGS based finishing diet contained high S (0.42 vs. 0.65%, DM). Likewise, Richter et al. (2012) observed that steers fed a corn DDGS based (40% DM) finishing diet contained 0.45% S tended to have lower feed intake than those fed a DDGS based finishing diet with 0.3 % S. In contrast, Boila and Golfman (1991) did not observe reduced feed intake in Holstein steers fed a finishing diet containing 0.39% S when compared to control steers fed a diet with 0.19% S. This might be due to the fact that the concentration of S (0.39%) in that diet was under the maximum tolerable level (0.4%) as set by NRC (1986) and therefore not high enough to exert an effect on feed intake.

The adverse impact of high dietary S on feed intake has been proposed to be due to suppressed ruminal motility. High dietary S results in elevated ruminal H_2S generation which inhibits rumen motility (Bird, 1972). In the present study, HS diets yielded higher ($P < 0.01$) ruminal H_2S concentrations relative to LS diets (Table 4.4). A negative correlation between high ruminal H_2S concentration and DMI has also been shown by Sarturi et al. (2011) and Uwituze et al. (2011a). Another reason for reduced DMI in heifers fed HS diets in the current study could partially be associated with palatability of the wheat DDGS. The wheat DDGS used in the DDGS offered to HS heifers contained almost 2 fold higher S than low S containing DDGS fed to LS heifers (1.07% vs. 0.55% DM) (Table 4.1). Elevated S content of the high S containing DDGS likely resulted from sulfuric acid added during the fermentation process, which may reduce its palatability. A negative impact of S concentration in corn DDGS on feed intake of feedlot steers was observed by Sarturi et al. (2011). They compared the effect of two different S

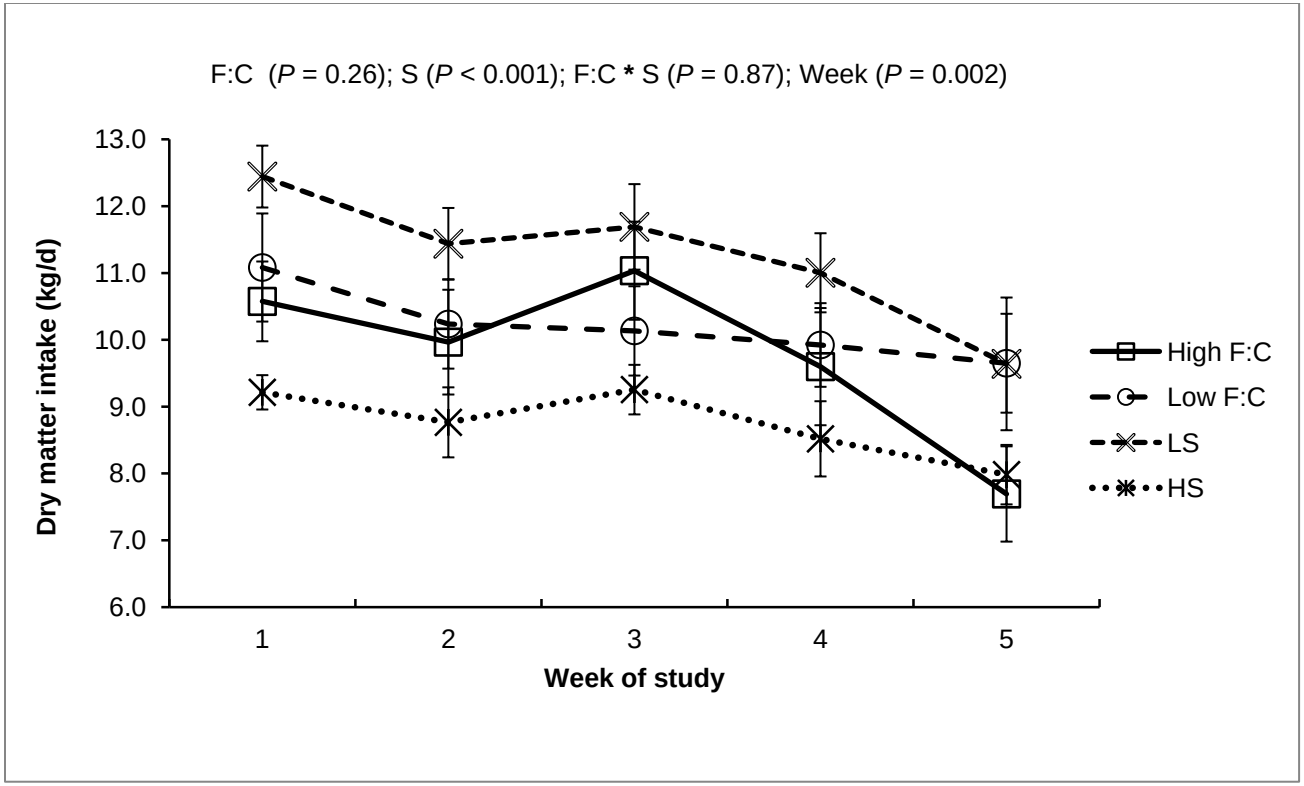


Figure 4.4. The effects of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on dry matter intake (DMI) of beef heifers

(0.82% vs. 1.16%) containing DDGS sources on feed intake and found high S containing DDGS resulted in lower DMI relative to low S containing DDGS.

4.4.3. Ruminal pH

There was no interaction ($P > 0.05$) between dietary S and F:C for all ruminal pH parameters (Table 4.3). Mean rumen pH was influenced by both dietary S ($P = 0.01$) and F:C ($P < 0.01$). Heifers fed low F:C diets exhibited reduced ($P < 0.01$) mean ruminal pH relative to those fed high F:C diets (pH = 6.02 vs. 6.30, respectively). Dietary S tended to decrease duration (min/d; $P = 0.10$) under the rumen pH threshold of 5.5; however, both of these pH parameters were increased by low F:C diets ($P \leq 0.04$). Heifers fed HS diets had a high ($P < 0.01$) maximum rumen pH and tended to have a high ($P = 0.08$) minimum ruminal pH. Both minimum ($P < 0.01$) and maximum ($P = 0.05$) rumen pH were lower for cattle fed low F:C diets. Despite the impact of day of study on DMI, it did not significantly impact ruminal pH ($P > 0.05$).

Increased rumen pH in steers exposed to high dietary S (0.65% vs. 0.42%) was observed by Uwituze et al. (2011a). They speculated that increased ruminal pH was associated with high dietary S would mainly due to the reduced feed intake. In the present study, heifers fed HS diets had reduced feed intake. Feed intake is a primary factor influencing ruminal pH (Nordlund, 2003), primarily through ruminal VFA production. It has been reported that high dietary S reduced total ruminal VFA concentration in steers fed a steam-flaked corn-based finishing diet (Uwituze et al., 2011a).

Rumen pH under the threshold value of 5.5 is used as an indicator of rumen acidosis (Penner et al., 2007). The mean rumen pH in all treatments was above 6.0. Heifers fed low F:C and LS diets experienced pH values below pH 5.5 for 2.7 and 2.3 h per day, respectively. Heifers

Table 4.3.The effect of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on ruminal pH in beef heifers

	Dietary treatments							
	F:C ratio		Sulfur			*P*-value		
	Low	High	High	Low	SEM[z]	F:C	Sulfur	F:C × Sulfur
Minimum pH	5.51	5.95	5.81	5.65	0.062	< 0.01	0.08	0.17
Mean pH	6.02	6.3	6.27	6.04	0.043	< 0.01	<0.01	0.60
Maximum pH	6.63	6.74	6.78	6.59	0.038	0.05	<0.01	0.90
Duration pH <5.5[y]	164	6	32	138	42.6	0.02	0.10	0.11
Area pH <5.5[x]	28.2	0.4	3.8	24.8	8.61	0.04	0.11	0.11

[z]SEM = pooled standard error of the mean; [y]Duration < 5.5 = min below 5.5 over 24h; [x]Area < 5.5 = min × pH below 5.5 over 24 h

fed high F:C and HS diets did not experience rumen acidosis.

4.4.4. Ruminal Sulfide, H_2S and Serum Sulfate Concentration

Results of ruminal S^{2-}, H_2S and serum sulfate concentrations are shown in Table 4.4. All three S metabolites were affected by dietary S ($P < 0.01$), but only ruminal S^{2-} was influenced by F:C ($P < 0.01$). There was no significant interaction ($P > 0.05$) between dietary S and F:C for these S metabolites.

High S diets produced about 4.5 fold higher ruminal H_2S gas concentration relative to LS diets ($P < 0.01$). Ruminal H_2S concentrations in heifers fed LS diets were under 500 ppm. Gould et al. (1997) found similar low ruminal H_2S concentrations in cattle fed low S containing finishing diets. The mean ruminal H_2S concentration for heifers fed HS diets was 2296 ppm. Gould et al. (1997) suggested that ruminal H_2S concentrations greater than 1000 ppm are potentially toxic and over 2000 ppm can precede the development of PEM. In the present study, there were no clinical signs of PEM observed despite the higher ruminal H_2S concentration. Likewise, Neville et al. (2010) reported that lambs exposed to elevated dietary S (0.65% or 0.83% DM) exhibited relatively high ruminal H_2S gas ranging from 2000 to 8000 ppm, but did not observe any clinical signs of PEM. They also did not see any clinical signs of PEM in cattle fed 20-60% DDGS-based finishing diets with S concentrations ranging from 0.6 to 0.9% (DM) despite significantly higher ruminal H_2S concentrations (Neville et al., 2012). Taken together, ruminal H_2S does not seem to be good indicator for assessing the risk of PEM in cattle. Ruminal H_2S concentrations did not vary ($P = 0.34$) between low F:C and high F:C diets. Hydrogen sulfide generation in the rumen is pH dependent. When rumen pH is low more H^+ is expected to be available to protonate S^{2-} and form H_2S (Gould, 1998). Although heifers fed low F:C diets had lower rumen pH, mean pH only differenced by 0.28 units from LS heifers. This pH

difference may not be large enough to affect ruminal H_2S concentration.

High sulfur diets resulted in greater ($P < 0.01$) ruminal S^{2-} concentration compared with LS diets (Table 4.4). Ruminal S^{2-} concentration was increased ($P < 0.01$) by low F:C and it did not differ with different sampling times ($P > 0.05$) within the day or between sampling days ($P > 0.05$).

It is not unexpected that high dietary S increased ruminal S^{2-} as all S compounds in the rumen are metabolised to sulfide (Lewis, 1954; Olkowski, 1992). Felix and Loerch (2011) found ruminal S^{2-} concentrations were greater for steers fed 0.43% S compared to those fed 0.25% S (DM).

Table 4.4. The effect of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on ruminal hydrogen sulfide (H_2S), ruminal sulfide (S^{2-}) and serum sulfate concentration in beef heifers

	Dietary treatments							
	F:C ratio		Sulfur			*P*-value		
	Low	High	High	Low	SEM[z]	F:C	Sulfur	F:C×Sulfur
Ruminal H_2S (ppm)	1297.7	1465.4	2296.3	466.8	122.29	0.34	< 0.01	0.31
Ruminal S^{2-} (μM)	2.14	0.96	2.20	0.90	0.201	< 0.01	< 0.01	0.19
Serum sulfate (ppm)	157.9	152.1	171.5	138.5	2.594	0.13	< 0.01	0.81

[z]SEM = pooled standard error of the mean

Unlike the other S metabolites evaluated, ruminal S^{2-} was increased by the high grain diet ($P< 0.05$). This may be due to reduced rumen pH which provides a more favourable condition for sulfate reducing bacteria to produce more sulfides in the rumen (Gould, 1998).

It is apparent that overall ruminal sulfide concentration was relative low compared with other studies (Gould et al., 1991; Suttle, 2010; Felix and Loerch, 2011). The critical lower sulfide levels in the rumen are reported to be about 1 ppm (Suttle, 2010). Our maximum ruminal sulfide concentrations were significantly lower than this critical value and were also much less than S levels in cattle fed a 0.25% S containing diet (Felix and Loerch, 2011). The reason for these

lower ruminal S^{2-} concentrations could be associated with the time of sulfide measurement after sample collection. We measured rumen samples for S^{2-} with a similar device to that of Gould et al. (1991) and Felix and Loerch (2011) However, in the present study, rumen S^{2-} was measured 2-3 h after sample collection, while others measured S^{2-}within 2 minutes after collection. Rumen S^{2-} has been reported to be unstable and can easily be converted to H_2S gas or incorporated into S containing AA by rumen microorganism; therefore, in the current study, S^{2-} may have been lost during the time between collection and analysis.

Sulfate is the end product of sulfide metabolism in the tissue, thus it was hypothesized that measuring serum sulfate can be an effective means of identifying cattle at risk of PEM. Serum sulfate concentration was 24% greater ($P < 0.01$) for HS heifers compared with LS heifers, which reflects dietary S intake. Our results are in agreement with Amat et al. (2012), who reported that serum sulfate levels in cattle fed DDGS based backgrounding or finishing diets reflected dietary S intake. It has also been reported that serum sulfate levels in cattle increased with increased S intake from feed (Weir and Rendig, 1954) or water (Hansard and Mohammed, 1969).

Surprisingly, serum sulfate levels were not affected by the F:C ($P = 0.13$). This is not in agreement with the results of trial 1 in Chapter 3. In the previous study, steers fed 40% corn or wheat DDGS based finishing diets (average 0.58% S) had lower serum sulfate levels compared to steers fed 17% corn or wheat DDGS based backgrounding diets (average 0.32% S). One potential explanation for such discrepancy with regard to the effect of diet on serum sulfate between these two studies may be due to the different mineral concentrations in the diet resulting from different inclusion rate of DDGS. In the present study, DDGS levels in both types of diets were similar (~38%). However, DDGS in the finishing diet in previous study was 23% higher

than in the backgrounding diet. Thus the mineral concentrations in finishing diets would presumably be higher than backgrounding diet as DDGS has relative higher minerals such as Mg, Mo, Cu, Se and Zn than barley grain or barley silage (Chapter 6). The higher the mineral concentrations in the diet, the higher amount of S would interact with these particular minerals in the rumen and form biologically unavailable complexes such as CuS, thiomolybdate, $MgSO_4$or other unknown complexes (Commack et al., 2010; Gooneratne et al., 2011). As a result, more of the dietary S would be affected by these interactions and become biologically less available, which may subsequently result in lower serum sulfate levels.

Serum sulfate was positively correlated with the ruminal H_2S ($r = 0.89$; $P < 0.01$).. Since PEM was not observed in the present study, it was not possible to correlating serum sulfate level with S-induced PEM. Future work should focus on establishing a correlation between serum sulfate and PEM.

4.4.5. Urine and Urinary Sulfate Output

Daily urine output tended to be reduced by high S ($P = 0.06$) and low F:C ($P = 0.07$) (Table 4.5). Neville et al., (2011) reported that urinary output in the lambs increased linearly with increasing DDGS which resulted in a linear increase in S intake. They ascribed this urinary output increase to increased water intake. They proposed that the increased water intake resulted from elevated S intake. Although water intake was not measured in present study, it can be seen from the urinary output results that dietary S did not have a major impact on water intake. Based on the results obtained from heifers fed similar levels of DDGS containing HS or LS diets in the present study, it can be concluded that the level of DDGS and not level of S in the diet may be a key factor that increased urinary output in the study of Neville et al. (2011). Increased DDGS will result in elevated CP concentration. Increased dietary CP concentration is reported to

increase urinary output in dairy cattle (Weiss and St-Pierre, 2010)

Urinary sulfate concentration in HS heifers was 131% greater than that of LS heifers. Heifers fed low F:C diets tended to have greater (P = 0.06) urine sulfate concentrations compared with those had high F:C diets. Heifers that consumed HS diets increased urinary sulfate output by a factor of 1.8 relative to heifers fed LS diets. Urinary sulfate output (62.49 vs. 66.85 g/d, Low vs. High F:C) was not influenced by the F:C (P = 0.49). Sulfur excretion from the body is primarily accomplished through urine (Underwood and Smitasiri, 1999). Sulfate is the major form of S in the urine (Suttle, 2010). Doyle and Moir (1979a) observed similar influence of dietary S on urinary S excretion in ruminants as in the present study. Neville et al. (2011) reported that lambs fed 0.84% S excreted 480% more S in the urine relative to those fed 0.22% dietary S. Our results supported the mechanism suggested by Neville et al., (2011), who proposed that ruminants on high S diets can have the ability to avoid S toxicity by increasing urinary S excretion.

Table 4.5. Effects of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on daily urine and urinary sulfate output of beef heifers

	Dietary treatments							
	F: C ratio		Sulfur			*P*-value		
	Low	High	High	Low	SEM[z]	F:C	Sulfur	F:C × Sulfur
Urine output (L/d)	11.3	14.0	11.3	14.1	0.96	0.07	0.06	0.52
Urinary sulfate (g/L)	5.94	4.87	7.54	3.27	0.350	0.06	< 0.01	0.11
Urinary sulfate output (g/d)	62.49	66.86	83.41	45.95	4.367	0.49	< 0.01	0.82

[z]SEM = pooled standard error of the mean

4.4.6. Short Chain Fatty Acid Absorption

Fractional rates of acetate, propionate and butyrate absorption were affected by both dietary S ($P < 0.05$) and the F:C ($P < 0.01$) (Table 4.6). High S diets reduced fractional rates of acetate, propionate and butyrate absorption by 40, 39 and 36%, respectively compared to LS heifers. While low F:C diets increased ($P < 0.01$) fractional rates of SCFA absorption. The overall fractional rate for SCFA absorption with the low F:C diet was 54% higher relative to high F:C diets.

Similar effects of dietary S and F:C on absolute disappearance rates of SCFA were observed with the exception that there was no significant effect of F:C on butyrate disappearance ($P = 0.11$) (Table 4.6). A stimulatory effect of grain on absorption rates of SCFA in cattle has been reported (Dirksen et al, 1985; Gabel et al., 1991), but not all studies have observed this response (Penner et al., 2009). An increased absorptive surface of rumen epithelium (Penner and Aschenbach, 2011) and reduced ruminal pH (Dijkstra et al., 1993) caused by the high grain diet could contribute to the increased SCFA absorption.

The most novel finding from SCFA absorption data was that dietary S inhibited SCFA absorption in cattle. The mechanism for how dietary S influences SCFA absorption is not clear. Although the relationship between high dietary S and SCFA absorption in ruminants has not been studied, adverse effects of sulfur on colonic acetate and butyrate oxidation has been identified (Moore et al., 1997; Roediger et al., 1997; Leschelle et al., 2005; Blachier et al., 2009). Moreover, sulfide-induced epithelial damage is used as a strategy to experimentally induce ulcerative colitis in monogastric animal models (Pitcher and Cummings, 1996). Thus, it could be expected that the outcomes of high dietary S for ruminants may induce similar pathogenesis in the ruminal epithelium. Sulfides are thought to inhibit butyrate oxidation in epithelial cells by

Table 4.6. The effect of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on short chain fatty acid (SCFA) absorption in beef heifers

	Dietary treatments							
	F:C ratio		Sulfur			*P*-value		
	Low	High	High	Low	SEM[Z]	F:C	Sulfur	F:C × Sulfur
Fraction rate of absorption (%/h)								
Acetate	41.4	23.3	21.8	36.4	6.12	0.007	0.036	0.56
Propionate	55.9	35.1	30.8	50.4	4.31	< 0.001	0.003	0.31
Butyrate	56.9	41.8	36.3	56.5	4.84	0.009	0.005	0.61
Absolute disappearance rate (mmol/h)								
Acetate	367.4	216.4	196.6	356.8	69.95	0.055	0.047	0.58
Propionate	256	166.6	141.4	248.4	29.54	0.012	0.010	0.33
Butyrate	110.4	82.4	69.5	115.8	13.85	0.109	0.017	0.45

[z]SEM = pooled standard error of the mean

inhibiting acyl-CoA dehydrogenation of activated fatty acids. This is an important rate-limiting step in the oxidation of SCFA (Bremer and Osmundsen, 1984; Moore et al., 1997). Inhibition of short chain acyl-CoA by sulfides is propsed to be caused by forming bytyrate CoA – persulfides (Shaw and Engel, 1987; Babidge et al., 1998) or by inactivating the electron transfer flavoprotein which is specific to the activity of short chain acyl – CoA dehydrogenase (Babidge et al., 1998). Inhibited activity of short chain acyl-CoA dehydrogenase will result in impaired β-oxidation of butyrate, which subsequently causes an energy deficiency state in epithelial cells (Roediger et al., 1993a). In addition, Blachier et al. (2009) proposed that since the inhibitory effect of sulfide on acetate oxidation is also identified, the inhibition of cytochrome c oxidation by sulfide rather than the inhibition of short chain acyl-CoA dehydrogenase might be major factor associated with SCFA oxidation inhibition in epithelial cells.

Reduced SCFA absorption from the rumen of cattle fed high dietary S might be due to the impaired SCFA metabolism in rumen epithelium. The rate of SCFA metabolism in epithelial cells is one of the important factors influencing the absorption of SCFA (Stevens and Stettler, 1966; Penner et al., 2011). Intraepithelial SCFA metabolism, particularly butyrate metabolism not only provides the majority of the energy to epithelial cells (Bergman, 1990), but also facilitates the absorption of SCFA by maintaining the concentration gradient between the cytosol and the lumen (Penner et al., 2011). The adverse impact of dietary S on SCFA absorption could be one of the contributors to poor animal performance in cattle associated with excessive S intake. Future study is needed to elucidate the mechanism underlying the negative impact of high dietary S on SCFA absorption.

In addition to high dietary S, reduced feed intake and increased ruminal pH would also be possible factors attributing to the reduced SCFA absorption in cattle fed HS diet. Reduced feed

intake would reduce the fatty acid production. Dijkstra et al. (1993) reported that acetate and propionate absorption across the reticulo-rumen epithelium in dairy cattle were reduced with an increase in their concentration. They also observed the negative impact of increased ruminal pH on ruminal absorption rate of acetate, propionate and butyrate.

4.5. Conclusion

High dietary S reduced DMI, urine output and SCFA absorption but increased the concentrations ruminal H_2S and serum sulfate. Urinary sulfate excretion and ruminal pH were also increased with high dietary S intake. Serum sulfate exhibited a positive correlation with ruminal H_2S. Sulfur metabolites were not affected by the F:C. Low F:C diet increased SCFA absorption. Composition of the diet does not seem to influence S metabolism in beef cattle.

5.0 EVALUATION OF SULFUR-THIAMINE INTERACTION IN HIGH DIETARY SULFUR EXPOSED OR SULFUR-INDUCED POLIOENCEPHALOMALACIA AFFECTED BEEF CATTLE

5.1. Abstract

This paper contains data from a metabolism trial and a field observation. The purpose of the metabolism trial was to evaluate the effects of dietary S concentration and F:C on thiamine and its phosphate esters in beef cattle. The purpose of the field observation was to determine brain thiamine metabolism in cattle affected by naturally occurring S-induced PEM. The metabolism trial was conducted as a randomized complete block using a 2 × 2 factorial treatment arrangement with main effects of F:C and S content using 16 ruminally cannulated heifers (initial BW 628 ± 48 kg). The F:C was modified by altering the proportion of barley silage (4% vs. 51%, DM basis), whereas, the S content was modified by using differing sources of wheat DDGS (~38%) to achieve low and high S diets (LS=0.3% vs. HS=0.67%, DM basis). Elemental S was also added to the pellets to increase the S content of the HS diets. Free thiamine, TMP, TPP from blood, brain, and rumen fluid were analyzed. No interactions between S concentration and F:C was detected. Low F:C increased ($P < 0.01$) ruminal free thiamine, TMP and TPP relative to high F:C. Blood and ruminal thiamine and its phosphate esters were not affected by HS ($P > 0.05$). Concentration of TPP was increased by 9.2% ($P = 0.10$) but with a concomitant numerical decrease in free thiamine in HS brains relative to LS brains. High S brains had greater TMP ($P < 0.05$) and total thiamine (free thiamine + TMP + TPP) ($P < 0.01$) than LS brains. No gross or microscopic changes indicative of PEM were detected in the brains of the experimental heifers. In the field observation study, brain thiamine and its phosphate esters were evaluated

from 4 S-induced PEM affected feedlot steers. These affected animals were treated with thiamine. They were exposed to high S intakes derived mainly from high S containing water (1755 ppm water SO_4) and secondarily from the feed (0.34% S). Thiamine levels from PEM brains were compared with the brains of heifers fed HS diets that were considered to be normal brains as they had no gross or microscopic changes indicative of PEM. The PEM brains had 36.5% lower TPP ($P < 0.05$) despite 4.9 fold higher free thiamine ($P < 0.01$) than normal brains. Considering the brain thiamine results from the experimental animals and field cases together, the results suggest that high dietary S intake may increase metabolic demand for TPP. Failing to supply enough TPP may lead to the development of malacic lesions in animals affected by S-induced PEM.

5.2. Introduction

Polioencephalomalacia is a nervous disease of cattle characterized by focal cerebral necrosis (Terlecki and Markson, 1961). This disease was first reported in North America by Jensen et al. (1956). Since the observation of the beneficial effect of thiamine administration to PEM in 1965, thiamine involvement in this condition has been recognized (Davies et al., 1965). Later research identified thiamine deficiency and altered thiamine metabolism as the major etiological factors of PEM (Loew and Dunlop, 1972; Thornber et al., 1979).

In the early 1980s, PEM outbreaks occurred in cattle herds fed high sulfate (> 2% sulfate DM) rations, which led to the development of a new hypothesis that excess S intake may be associated with PEM (Raisbeck, 1982). This hypothesis was further supported by Gooneratne et al., (1989b); and Gould et al. (1991), who experimentally induced PEM in sheep and cattle, respectively, by feeding high S containing diets. Since these studies, numerous field cases of

PEM associated with excess S intake derived either from feed or water, or both have been reported in cattle (Cummings et al., 1995a; Loneragan et al., 1998; Niles et al., 2000; Kul et al., 2006; Knight et al., 2008; Richter et al., 2012). Although S-induced PEM has been recognized for 3 decades, the role of S in PEM remains unclear (Olkowski, 1997).

Cattle affected by S-induced PEM frequently respond to thiamine treatment (Harries, 1987; Beke and Harionaka, 1991). This suggests a possible causal relationship between excess S and thiamine on the development of malacic lesions. An interaction between excess dietary S and thiamine in ruminants was first noted by Goetsch and Owens (1987) who observed that high dietary S reduced the amount of thiamine passing from the rumen in dairy steers. Reduced blood thiamine in feedlot cattle exposed to excess dietary S was also observed by Gooneratne et al. (1989b) and Olkowski et al. (1991). Thiamine supplementation reduced the incidence of PEM in lambs fed high dietary S (Rousseaux et al., 1991). Following these observations, increased thiamine destroying activity (Olkowski et al., 1993a) and reduced thiamine synthesis (Alves de Oliveira et al., 1997) in the rumen due to increased dietary sulfate were demonstrated in vitro. The thiamine destroying effect of S is attributed to sulfite which can cleave thiamine (Leichter and Joslyn, 1969). Paradoxically, elevated blood thiamine in lambs fed high dietary S has also been reported (Gooneratne et al., 1989a; Olkowski et al., 1992).

Although information on the causal relationship between S and thiamine is available, information regarding the effect of high dietary S on thiamine phosphorylation in beef cattle is limited. Thiamine is present in mammalian tissues in four different forms such as free thiamine, TMP, TPP, and TTP. There is a relationship between these compounds as free thiamine is converted to TPP through a phosphorylation process. Thiamine pyrophosphate is dephosphorylated to TMP, forming an intermediate TTP. Finally, TMP is hydrolyzed to

thiamine which is then converted to TPP directly (Tallaksen et al., 1992; Liu and Hurley, 2011). Thiamine pyrophosphate is the only active form of thiamine. It plays a fundamental role in energy metabolism as it is a cofactor for major enzymes in glucose metabolism and the TCA cycle (Lindqvist et al., 1992; Bettendorff et al., 1996; Widmann et al., 2010). Any alteration in TPP synthesis or supply is often associated with neurological disorders such as Wernicke-Korsalkoff syndrome (Butterworth, 2003) and Alzheimer's disease (Kish et al., 1999) of humans. Thus, determining the effect of S on thiamine phosphorylation is important to understand the pathogenesis of the development of PEM lesions. An adverse effect of S on thiamine phosphate esters in microorganisms and body cells has been reported (Belitz et al., 2009). This led to the hypothesis that high dietary S may influence thiamine phosphorylation in cattle. Furthermore, considering the fact that thiamine is cleaved by sulfite (Leichter and Joslyn, 1969) and thiamine is destroyed by thiaminase (Brent, 1976), which are both pH dependant, it was hypothesized that the effect of high S on thiamine metabolism may be influenced by dietary forage-to-concentrate ratio. To test these hypotheses, the effects of dietary S concentration and F:C on ruminal, blood, and brain thiamine and its phosphate esters in beef heifers were evaluated. In addition, given the fact that administration of thiamine in early stages of S-induced PEM results in improvement of clinical status of some animals, but is totally ineffective in others, it is possible that those animals that do not respond to thiamine treatment may have some defect in thiamine metabolism. Hence thiamine and its phosphate esters in the brains of S-induced PEM affected steers were also evaluated.

5.3. Material and Methods

Animal care, experimental design, diet composition and sampling regime for metabolic trial were described in Chapter 4.

5.3.1. Sampling

Feed and water sampling: Feed and water sampling from metabolic trial were described in Chapter 4.

Rumen fluid and blood sampling: Rumen and blood samples were taken at 1200 h on d 1, 7, 10, 14, 21, 28, and 35 of the experimental period. The procedures of sampling are referred to Chapter 4. Thirty mL of rumen liquid sample was collected and immediately flash frozen with liquid nitrogen. Ten mL of blood samples were collected with green top vacutainer tubes (BD Vacutainer®, Ref 367880) contained lithium heparin. Collected samples were stored at -80 ^{0}C for the analysis of thiamine and its phosphate esters.

5.3.2. Brain Tissue Sampling and Pathological Examination

The heifers were humanely euthanized using an over-dose of Phenobarbital (Euthanyl-Forte) and immediately transported to the post-mortem room at the Western College of Veterinary Medicine, Saskatoon, SK. Brain tissues from 16 experimental heifers were collected for gross and histopathological examinations of classic gross or microscopic changes indicative of PEM as well as for analysis of thiamine and its phosphate esters. Intact brains were removed and equally divided into two halves. One half was fixed with 10% formalin and stored for gross and histopathological examination. The other half was stored at –80ºC for thiamine and its phosphate ester analysis. In addition, brains from 4 PEM affected cattle were collected, stored and processed in the same manner as the experimental brains.

Both gross and histopathopogical examination on brains were performed by a pathologist from the Department of Veterinary Pathology, University of Saskatchewan (Saskatoon, SK). Each brain was cross-sectioned (ranging from 10 to 15 per brain) and all cross sections were

examination under ultraviolet (UV) light for the presence of fluorescence. Following the UV light observation, the following regions; parietal cortex, occipital cortex and medulla were taken for histological examination. The formalin-fixed brain tissues were embedded in paraffin, sectioned to a thickness of 5µm, stained with hematoxylin-eosin and examined by light microscopy.

History of clinical cases: Brain thiamine and its phosphate esters were also evaluated from 4 naturally occurring PEM cases in feedlot steers. In December 2011, a PEM outbreak occurred at a farm located near Moose Jaw, Saskatchewan, Canada. This farm had about 1040 steers (Charlais crossbreds) and purchased between October 20 and November 3, 2011. These cattle were housed in 7 pens. Each pen had 150 animals except for one that had only 80 animals. Cattle were fed free choice pellet (barley, lentil screenings with monensin and mineral). Free choice hay was also offered. Water was from a deep well. In early December, about 18 animals exhibited clinical signs of neurological disease including progressive blindness, head pressing, and staggering. These affected animals were from two pens. The clinically affected animals were initially treated with Dexamethasone and a combination of florfenicol and flunixin meglumine (Resflor, 6 ml/45kg SQ, Merck Animal Health), and later treated with trimethoprim-sulfamethazine and thiamine (Thiamine Hydrochloride Injection U.S.P., 100 mg/mL thiamine hydrochloride, 2 ml/45 kg). Four of the affected animals did not respond to thiamine treatment and either died or were humanely euthanized, while the rest of the affected animals responded to thiamine treatment and recovered. Brain tissues from the 4 animals that did not respond to treatment were collected. After the confirmation of PEM by gross and histopathological examination, feed (not including hay) and water samples from each pen were collected for total S analysis.

5.3.3. Chemical Analysis

Feed and water sample analysis: Feed samples were analyzed for CP, starch, ADF, NDF, Ca, P, Na, K and S by Cumberland Valley Analytical Services (CVAS, Hagerstown, MD). Water sulfate was determined using Inductivity ICP-AES by Saskatchewan Research Council Analytical Laboratory (Saskatoon, SK). Sulfur concentration of feed samples collected from the PEM affected feedlot was analyzed by Agri-Food Laboratory of University of Guelph (Guelph, ON).

Rumen free thiamine, TMP and TPP analysis: Free thiamine, TMP and TPP were measured from rumen fluid samples by High Performance Liquid Chromatography (HPLC) using a method modified from Olkowski and Classen (1996). One mL of rumen fluid sample was placed into a 5 mL culture tube (VWR, cat # 211- 0066). Then 3 mL 0.7 *M* perchloric acid was added at a ratio 1:3 v/v followed by centrifugation at 2526 g for 10 min. The supernatant was adjusted with 20% KOH and 10% KH_2PO_4 phosphate to maintain the final pH between 6 and 6.5. The supernatant was then centrifuged at 3000 g for 5 min. After which the supernatant was filtered through 0.45 µm filter (WhatmanPuradisc^{TM} 13 mm syringe filters) prior to transfer to brown HPLC vials for HPLC analysis. Authentic standards from Sigma were used for the standardization. A mixture of free thiamine, TMP and TPP standards were prepared from stock solutions and were subjected to the same procedure as the sample. A 1100 Series HPLC system (Agilent Technologies Canada Incorporated, Mississauga, ON) was used. Instrument separation and data analysis were controlled by ChemStation™ LC-3D software. Separation of the analytes was performed with a polymeric reverse phase column (PRP-1 5 µm 100 Å 4.6 × 250 mm PEEK 79571, Hamilton, Reno, Nevada, USA). The mobile phase consisted of 90% 25 m*M* $KHPO_4/KH_2PO_4$ buffer adjusted to pH 5.8-5.9, and 10 % HPLC grade methanol. Mobile phase

was filtered with 0.20 µm membrane filter (Chromatographic Specialties, CSMF47N20) and then degassed under vacuum. The mobile phase was delivered at a flow rate of 1 mL/min by quaternary pump (model G1311A) and the sample was injected at a rate of 50 µl by an auto-sampler (model G1329A). Post column derivatization was used. $K_3Fe(CN)_6$ dissolved in aqueous NaOH (3 *M*) was used as an oxidizing reagent. This reagent was kept in a dark glass bottle at room temperature. The oxidation reagent was delivered at a rate of 0.10 mL/min by an isocratic pump. The post column reactor consisted of a zero-dead volume tee-piece and a Teflon capillary (100 cm), wound to form a mixing coil. Detection was performed by a fluorescent detector (FLD, model G1321A) at the excitation wavelength of 360 nm and emission wavelength of 460 nm. The peak area was plotted and integrated manually. Each sample was analyzed in duplicate. The extraction of analytes from the sample was tested by extracting the pellets remaining from first extraction. No detectable amounts of analytes were found in these pellets. Calibration curves were constructed for each run from seven different concentrations of mixed standards namely 2.5, 5, 10, 20, 40, 80 and 160 ng/mL. All calibration curves constructed during the analysis produced considerably high linearity with the regression coefficient higher than 0.999 for the calibration curves of all analytes.

Blood free thiamine, TMP and TPP analysis: Free thiamine, TMP and TPP were tested from the whole blood sample using HPLC method. All the procedures of blood thiamine analysis were similar to the rumen fluid sample analysis with the following exceptions. The mobile phase was 94% of 25m*M* $KHPO_4/KH_2PO_4$ and 6% of HPLC grade methanol. The mobile phase was delivered at a flow rate of 1.2 mL/min and the analytical column used was PRP-1 5 µm 150 × 4.1 mm 79444 (Hamilton, Reno, Nevada, USA). The oxidation reagent was delivered at a rate of 0.12 mL/min. Calibration curves were constructed for each run from seven different

concentrations namely 0.625, 1.25, 2.5, 5, 10, 20 and 40 ng/mL. All calibration curves constructed during the analysis produced high linearity with the regression coefficient higher than 0.999.

Brain free thiamine, TMP, TPP analysis: Thiamine, TMP and TPP were analyzed from the brain tissues using HPLC method. About 2 to 3 g (wet) of brain tissue was sectioned from occipital region and finely minced. Subsequently115 to 145 mg of tissue was placed into a 5 mL homogenizer tube. Then 0.7 *M* perchloric acid was added to the aliquots 1 mL/100 mg tissue. The rest of the procedures were the same for the blood sample analysis with the following exceptions. Two different mobile phases were used: first mobile phase contained 100% 25m*M* $KHPO_4/KH_2PO_4$, and second one contained 94% of 25 m*M* $KHPO_4/KH_2PO_4$ and 6% of HPLC grade methanol. The first mobile phase was introduced in the first five min and then the second mobile phase was delivered. Calibration curves were constructed from 6 different concentrations such as 1.25, 2.5, 5, 10, 20 and 40 ng/mL. All calibration curves constructed during the analysis produced high linearity with the regression coefficient higher than 0.999. Brain tissues were analyzed in triplicate.

5.3.4. Statistical Analysis

Blood and rumen fluid data were analyzed as a randomized complete block (RCB) design with repeated measures with 2 × 2 factorial treatment arrangement using the Mixed Model Procedure of SAS(Version 9.2; SAS Institute Inc. Cary, NC). No significant block effect was observed as such the block was removed from the model and data analyzed with the following model: fixed effects of F:C, S, F:C × S, day, F:C × day, S × day and F:C × S × day, and random effect of animal nested within the block. The covariance error structure for each model was chosen based on its lowest AIC value. Brain thiamine data from experimental heifers were

analyzed as RCB design with a 2 × 2 factorial treatment arrangement. After observing no significant block effect, the block was removed from the model and the final model included fixed effects of F:C, S and F:C × S, and random effect of animal nested within the block. The Sattterthwaite method was applied to determine the degrees of freedom. Means were compared using Tukey's multiple comparison test. Differences in TPP and TMP between PEM and HS brains were compared with 2-tailed t-test using the Mixed Model Procedure of SAS; while differences in free and total thiamine between PEM and HS brains were compared with the Mann-Whitney test. Significance was declared at $P < 0.05$ and trends were discussed when $0.05 \leq P \leq 0.10$.

5.4. Results

5.4.1. Sulfur Intake

Actual S concentrations in LS and HS diets averaged 0.31 and 0.62% (DM basis), respectively (Chapter 4, Table 4.1). The actual mean S concentration in HS diets was 0.05% lower than formulated value. This discrepancy resulted from slightly lower S content of the supplements to which elemental S was added during pelleting. Daily S intake was greater ($P < 0.01$) for heifers fed HS diets relative to those fed LS diets (53.0 vs. 34.5 g/d, respectively). As expected, no difference ($P = 0.53$) in daily S intake was observed between heifers fed high F:C and low F:C diets (44.4 and 43.0g/d, respectively) (Chapter 4, Figure 4.3). Water S intake was not estimated however sulfate content of the water used in this trial (110 mg/L) was low (Olkowski, 2009).

Sulfur content of the feed offered to the cattle from which the field cases of PEM were obtained was 0.34% (DM). Sulfate concentration in water was 1755 ppm, a value almost three fold higher than the recommended sulfate level (< 600 ppm) for feedlot cattle (NRC, 2005). Total S intake

from feed and water was estimated. Water intake was estimated according to the equation for feedlot steers that has been developed by Hicks et al. (1988). The feed intake was estimated to be 10 kg/d/head (DM). Estimated daily S intake for cattle in this feedlot was 47 g/d/head, which was similar to the experimental heifers fed HS diets ($P > 0.05$).

5.4.2. Gross Pathology and Histopathology of Brain Tissue

Experimental animals: There were no gross changes under regular or UV light indicative of PEM or any other pathological process in brains of experimental animals. Histopathological examination did not reveal any changes consistent with PEM. Occasional subtle to mild perivascular lymphocytic infiltration was observed in white matter or meninges in a few sections. However, they were considered to be incidental findings and their clinical relevance is not clear.

Field cases of PEM: All affected brains showed typical gross and microscopic changes consistent with PEM. There were grossly evident cerebrocortical lesions, including cortical ribbons of autofluorescence under UV light in all affected brains. Histopathoplogical examination showed severe neuronal necrosis and perinueuronal vacuolation in the cerebral laminae. According to the gross and histopathological findings, as well as water and feed S content, etiology of these field cases was determined as S-induced PEM.

5.4.3. Ruminal Thiamine and Its Phosphate Esters

In the present study, free thiamine, TMP and TPP were measured. Total thiamine reported in the present paper was obtained by adding these three forms of thiamine. No interaction was observed between dietary S and F:C for ruminal thiamine and its phosphate esters ($P > 0.05$) (Table 5.1). Ruminal TPP, TMP and free thiamine concentrations in cattle fed

Table 5.1. The effect of dietary sulfur and forage-to-concentrate ratio (F:C) on thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), free thiamine and total thiamine concentration in the rumen, blood and brain tissue of beef heifers

	Dietary treatments							
	F:C		Sulfur			*P*-value		
	Low	High	High	Low	SEM[z]	F:C	Sulfur	F:C × Sulfur
Rumen (ng/mL)								
TPP	310.9	124.7	215.1	220.5	21.97	< 0.001	0.87	0.81
TMP	153	30.9	91.3	92.6	15.94	< 0.001	0.96	0.4
Free thiamine	319.9	102	187.5	234.9	27.07	< 0.001	0.24	0.47
Total thiamine	785.1	258.1	495	548.1	54.29	< 0.001	0.50	0.83
Blood (ng/mL)								
TPP	14.2	13.8	13.9	14.1	0.82	0.71	0.9	0.18
TMP	9.1	7.4	8.1	8.4	0.49	0.37	0.64	0.62
Free thiamine	6.2	5.6	5.7	6.1	0.5	0.02	0.65	0.88
Total thiamine	30.1	26.7	28.2	28.6	1.45	0.07	0.96	0.43
Brain (ng/g, wet wt)								
TPP	488.8	493.9	512.9	469.8	17.14	0.84	0.1	0.55
TMP	607	631.5	656.1	582.4	17.95	0.35	0.01	0.95
Free thiamine	206.3	193.9	192.4	207.8	10.43	0.42	0.32	0.76
Total thiamine	1291.9	1318.8	1361.3	1249.4	20.62	0.37	< 0.01	0.46

[z]SEM = pooled standard error of the mean

low F:C diet were increased by 149.8, 349.9 and 213.8%, respectively, compared to cattle fed high F:C diet. Dietary S, however, did not influence the concentrations of TPP (P = 0.87), TMP (P = 0.96), free thiamine (P = 0.24), and total thiamine (P = 0.50) in the rumen.

5.4.4. Blood Thiamine and Its Phosphate Esters

There was no interaction between dietary S and F:C for blood thiamine and its phosphate esters ($P > 0.05$) (Table 5.1). Blood total thiamine ($P = 0.07$) and free thiamine ($P = 0.02$) were increased by low F:C. However, blood TPP ($P = 0.71$) and TMP ($P = 0.37$) concentrations were not affected by F:C. There was no dietary S effect ($P > 0.05$) on blood thiamine and its phosphate esters.

5.4.5. Brain Thiamine and Its Phosphate Esters

Experimental cattle: No interaction ($P > 0.05$) between dietary S and F:C was observed for brain thiamine and its phosphate esters (Table 4.2). Brain thiamine and its phosphate esters were not influenced by F:C ($P > 0.05$). However, HS brains had greater total thiamine ($P < 0.01$) relative to LS brains. Thiamine pyrophosphate ($P = 0.10$) and TMP ($P = 0.01$) in HS brains were increased with concomitant numerical decrease in free thiamine ($P = 0.35$).

Field cases of PEM: Brain thiamine and its phosphate esters in PEM cattle were compared with the experimental cattle fed HS diets. Although the experimental cattle did not show any clinical and histopathological features indicative of PEM, cattle fed HS diets were compared with PEM as clinically normal cattle because their S intake was similar to PEM affected cattle. The PEM brains had 36.5% lower ($P = 0.04$) TPP despite a 4.9 fold higher free thiamine ($P < 0.01$) relative to normal brains (Figure 5.1). Total thiamine ($P = 0.10$) and TMP

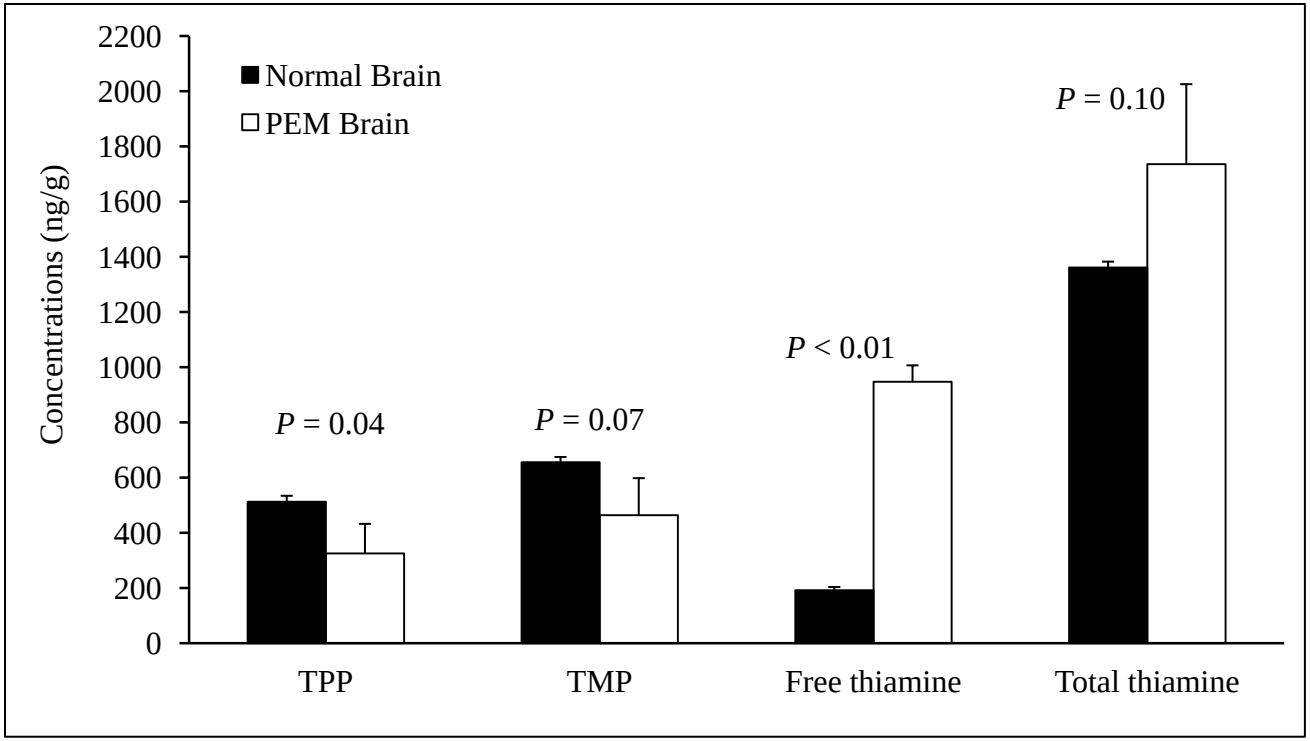


Figure 5.1 Comparison of brain thiamine and its phosphate esters between normal (n = 8) and sulfur-induced PEM affected (n=4) cattle (ng/g, wet wt)

(P = 0.07) tended to be lower in PEM brains relative to normal brains.

5.5. Discussion

5.5.1. Ruminal Thiamine and Its Phosphate Esters

Total thiamine in the rumen is of dietary and microbial origin with the majority of total thiamine (> 90%) present in the rumen fluid and less than 10% is contained in the microbes and small feed particles (Breves et al., 1980). Thiamine synthesis can be influenced by the F:C (Lardinois et al., 1944; Miller et al., 1986). It has been reported that fed a forage based diet failed to meet their thiamine requirements, while animals fed high grain diets usually managed to synthesize adequate thiamine for their needs (Breves et al., 1980). In the present study, low F:C significantly increased total ruminal thiamine relative to high F:C. Similar to our results, Hayes et al. (1966) reported that thiamine concentration was greatest in the rumen fluid of steers fed all concentrate diets. The positive effect of low F:C on ruminal thiamine status may due to the following: 1) a high grain diets may provide a greater amount of readily fermentable carbohydrate that can stimulate thiamine synthesis (Lardinois et al., 1944); 2) high grain diets may provide greater exogenous thiamine as cereal grain contains higher thiamine than forages (NRC, 2000). Thiamine synthesis is reported to be positively related to the duodenal flow of microbial protein and VFA concentration. High grain diets can stimulate both ruminal passage rate of the microbial protein (Firkins, 1996) and VFA production in the rumen (Suárez et al., 2006). However, the effect of F:C on thiamine synthesis was not observed in dairy calves fed different ratios of grain to hay when good quality grass hay was offered. Whereas thiamine synthesis was stimulated when high quality grass hay was replaced with poor quality (matured) hay (Conrad and Hibbs, 1954).

Although total thiamine status has been evaluated in the rumen of cattle and sheep fed

different diets, the effects of F:C on ruminal free thiamine and its phosphate esters had not been studied in vivo prior to this trial. Our results indicate that ruminal TPP, TMP and free thiamine concentrations were increased by the high grain diet.

In the present study, the destructive effect of S on thiamine in the rumen was not observed. However, it has been reported elsewhere. The adverse effect of dietary S on ruminal thiamine was first reported by Goetsch and Owens (1987), who observed that high dietary S reduced the amount of thiamine passing through the rumen in dairy cattle. Olkowski et al., (1993a) demonstrated the thiamine destroying effect of S in vitro. They reported that 40-60% of the added thiamine to the rumen contents was destroyed by the addition of sulfate. However, the effect of S on thiamine synthesis in the rumen is controversial. Olkowski et al. (1993a) did not observe an alteration in thiamine synthesis by sulfate addition in the rumen content in vitro. Likewise, high S did not impair thiamine synthesis in sheep fed a semi-synthetic and thiamine–free diet (Alves de Oliveira et al., 1996). In contrast, Alves de Oliveira et al. (1997) observed suppressed ruminal thiamine synthesis in vitro with increased sulfate addition.

The proposed destroying effect of high S on ruminal thiamine is attributed to sulfite ions (Olkowski et al., 1993a; Olkowski, 1997). Sulfite can cleave the thiamine molecule into biologically inactive compounds (Leichter and Joslyn, 1969; Brent and Bartley, 1984). Sulfite is a key intermediate produced during the reduction of sulfate to sulfide in the rumen (Lewis, 1954). Ruminal sulfate originates from the diet and from sulfate recycled back to the rumen (Kennedy et al., 1975). Hence a sustainable amount of sulfate could maintain generation of sulfite in the rumen which can interact with thiamine (Olkowski, 1997).

The rate of thiamine cleavage by sulfite ions is pH dependent (Leichter and Joslyn, 1969; Lenz and Holzer, 1985). Thiamine cleavage by sulfite is increased dramatically with a pH from 4

up to 5.5 to 6.0 and then decreased sharply (Leichter and Joslyn, 1969). Considering this, it is plausible that rumen pH may influence the thiamine cleavage effect of sulfite in the rumen. A more acidic rumen environment (pH < 6) may provide favourable conditions for thiamine cleavage of sulfite. In the present study, cattle fed HS diets had mean rumen pH of 6.27, which was not within the pH range for thiamine cleavage by sulfite. Therefore rumen pH of HS cattle was less likely to provide favourable rumen conditions for thiamine cleavage by sulfite. This could be one of the possible reasons why no detrimental effect of S on thiamine was observed in the present study. Future studies should evaluate the specific effect of ruminal pH on thiamine destroying effect of S in the rumen.

In addition, type of S compounds entering the rumen could influence thiamine destroying effect of S. In the present study, the majority of S entering the rumen originated from wheat DDGS. Sulfur in DDGS is present in both organic (cysteine and methionine) and inorganic (mainly sulfate) forms (Zhang, 2010). Since sulfite is generated during the reduction of sulfate into sulfide in the rumen, only the inorganic form of S in DDGS may be involved in sulfite generation. Therefore, S derived from DDGS may not be able to generate as high sulphite levels as other S sources such as $NaSO_4$ which was the major S source used in studies that observed thiamine destructive effects of S (Olkowski et al., 1993a; Alves de Oliveira et al., 1996; Alves de Oliveira et al., 1997).

Dietary S did not influence ruminal TPP and TMP levels. Although information regarding the destructive effect of S on thiamine phosphate esters in the rumen is limited, the cleavage of thiamine phosphate esters has been demonstrated in vitro, microorganisms (Lenz and Holzer, 1985) and in body cells (Belitz et al., 2009). Lenz and Holzer (1985) observed that TPP and TMP in yeast were cleaved by sulfite. Cellular TPP has also been reported to be reduced by

sulfite via either inhibiting TPP synthesis or enhancing degradation or both (Belitz et al., 2009). The reasons discussed above as to why there was no observe the effect of S on total thiamine may account for why TPP and TMP were not influenced by high dietary S.

5.5.2. Blood Thiamine and Its Phosphate Esters

Whole blood thiamine is known to reflect both intracellular and extracellular thiamine (Tallaksen et al., 1992; Tallaksen et al., 1993). Therefore, whole blood was used in the present study to evaluate blood thiamine levels. Total blood thiamine in normal cattle and sheep is reported to range from 23-56 ng/mL (Hill, 1969). Based on this range, cattle in any of the treatment groups did not experience thiamine deficiency (total thiamine without TTP, 27-30 ng/ml).

Surprisingly, cattle fed low F:C only had 12.5% higher (P = 0.07) total blood thiamine relative to cattle fed high F:C, despite having twice the total ruminal thiamine. This indicates that the amount of thiamine getting into the blood from the rumen with low F:C diets was low. Thiamine is mainly absorbed from small intestine (Miller et al., 1986), not through the rumen wall (Hoeller et al., 1977). Miller et al., (1986) observed that duodenal concentrations of thiamine in steers did not differ between high-concentrate (HC) (88.9% of corn) and low-concentrate (LC) diets (29.5% corn) despite greater thiamine content in HC the diet (2.54 vs.1.86 µg/g, DM). Free thiamine in the blood of low F:C cattle was significantly greater in comparison with cattle fed high F:C, which reflects free thiamine in the rumen. Free thiamine is the only absorbed form of thiamine (Rindi and Laforenza, 2000). Blood TPP and TMP levels did not differ with F:C, which indicates that the F:C is less likely to influence blood thiamine phosphate esters.

One possible reason for this greater loss of ruminal thiamine in cattle fed low F:C diets

could be explained by greater TPP utilization by rumen microorganism. Ruminal TPP in low F:C cattle was 2.5 fold higher than high F:C cattle. Since TPP in the rumen is utilized by rumen bacteria (Alves de Oliveira et al., 1997), the greater proportion of total thiamine in the rumen of low F:C cattle could actually be utilized through the rumen bacterial metabolism of TPP. Furthermore, malabsorption of thiamine cannot be ignored as one of the factors that may lead to the low F:C cattle having no significantly greater amount of total blood thiamine despite significantly greater ruminal thiamine. The greater disappearance of ruminal thiamine in low F:C cattle may also have resulted from the activity of thiaminase in the rumen. However, the amount of thiamine destroyed by thiaminase would be limited in the low F:C diet as it did not provide acidic rumen condition which could stimulate thiaminase production (Brent, 1976).

The results of the present study demonstrated that blood thiamine and its phosphate esters were not influenced by the high S diet. Since there was no effect of dietary S on ruminal thiamine, it is not surprising to see that blood thiamine did not differ among HS and LS cattle. The effect of dietary S on blood thiamine is debatable. High dietary S was reported to depress blood thiamine concentration in steers (Goetsch and Owens, 1987). Gooneratne et al. (1989b) observed blood thiamine deficiency in a S-induced PEM affected cow. However, blood thiamine in PEM affected animals was reported to be increased (Gooneratne et al., 1989a; Gould et al., 1991; Olkowski et al., 1992). In the present study, animals exposed to high S were all clinically normal, thus the blood thiamine levels would not be expected to be elevated as in PEM affected animals.

5.5.3. Brain Thiamine and Its Phosphate Esters

Experimental cattle: Thiamine plays a fundamental role in maintaining normal function of brain. Thiamine pyrophosphate is the active form of thiamine and is a cofactor for several

enzymes that regulates glucose metabolism (Harper, 2006). Insufficiency of TPP in the brain is associated with neurological disorders as it largely depends on glucose metabolism to meet its energy metabolism (Butterworth, 2003). In the present study, there was an increase in TPP, TMP and total thiamine in HS brains relative to LS brains. This suggests that animals exposed to high dietary S may have higher metabolic demand for thiamine. Assuming equal supply, the increased TMP and numerically lowered free thiamine in HS brains, suggests that heifers exposed to high S may satisfy their brain TPP requirement by converting more free thiamine into its phosphate esters.

Increased blood thiamine has been seen in animals that have developed S-induced PEM (Gooneratne et al., 1989a; Gooneratne et al., 1989b; Gould et al., 1991; Olkowski et al., 1992). Thiamine supplementation prevented animals exposed to excess S from the developing clinical sings of PEM (Olkowski et al., 1992). The beneficial role of thiamine administration in cattle affected by S-induced PEM is well recognized (Harries, 1987; Beke andHarionaka, 1991). Based on these facts, it has been suggested that brain thiamine may increase in cattle exposed to excess S in order to protect the brain tissue from increased S toxicity by scavenging sulfide or sulfite ions (Olkowski, 1997). The increased thiamine in HS brains in the present study fits this hypothesis. Another possible factor for the increased thiamine and TPP in the HS brains might be that sulfide or sulfite toxicity may result in pathologically increased energy consumption. The increased energy consumption may lead brain tissue to require more TPP to meet its increased energy demand.

Different F:C fed in the present study did not influence thiamine and its phosphate ester levels in the brain. This suggests that brain thiamine metabolism is not altered by the nature of the diet.

Field cases of PEM: Results of the comparison in brain thiamine between clinically normal experimental cattle and PEM cattle indicated that there was a significant reduction of TPP in PEM brains regardless of the fact that free thiamine level was elevated. This suggests that the affected animals were unlikely to meet their demand for TPP. To our knowledge, this is only the second study that has reported thiamine and its phosphate esters in S-induced PEM affected ruminants. Gould et al. (1991) evaluated thiamine and its phosphate esters (TPP and TMP) from the brain tissues of dairy calves (n = 3; initial BW 145 kg) affected by experimentally S-induced PEM. Although they did not see a statistical reduction in thiamine and its phosphate esters, the calves which developed PEM had 6% reduced TPP in comparison with 3 clinically normal, non-experimental steers. These results, although not significant, point to a numerical reduction of TPP in PEM affected calves. Considering the data from the HS brains, a small reduction in TPP in the affected calves reported by Gould et al. (1991) and the suppressed brain TPP level in the animals from the field case, it can be speculated that there may be a continuous increase in the demand for TPP as S intake increases. Once the increase in TPP demand reaches a certain point, the host may not be able to maintain enough supply of TPP to meet their ever increased demand. This may subsequently lead to the development of PEM.

Deficiency of TPP usually leads to suppressed thiamine dependent enzyme activity. Decreased thiamine dependent enzyme activity is considered one of the major factors causing several neurological disorders such as WKS (Butterworth, 2003) and AD (Kish et al., 1999) and Parkinson's disease (Mizuno et al., 1994a), all commonly seen in humans. Based on the studies with PEM in lambs fed thiamine free diets (Thornber et al., 1979), as well as the observation of a significant reduction of TPP in thiamine deficient lambs (Thornber et al., 1981), it has been proposed by these authors that only a slight decrease in TPP level could cause malfunction of the

brain, and the moderate reduction of TPP could lead to develop malacic lesions. Taken together, it is possible that metabolic TPP deficiency may be involved in the development of PEM lesions in cattle exposed to high dietary S.

Thiamine data from the field case also sheds light on a possible explanation for the lack of response from some PEM affected cattle to thiamine treatment. Thiamine treatment is the only recognized treatment for S-induced PEM. Most animals responded to a high dose of thiamine (Harries, 1987; Beke and Harionaka, 1991), while some animals, as with the present field cases, do not respond (Jeffrey et al., 1994; Bulgin et al., 1996). The lack of response to thiamine treatment has been proposed to be due to the fact that brain lesions were advanced beyond clinical recovery (Olkowski, 1997). Thiamine status in PEM brains, however, indicated that the lack of thiamine treatment response may be owing to metabolic weakness in converting free thiamine into TPP. This is based on the observation that there was elevated free thiamine in PEM brains but reduced TPP concentrations. Thus, it is speculated that animals that do not respond to thiamine treatment may have a defect in thiamine metabolism which could inhibit the conversion of free thiamine into TPP. Inhibition of TPP synthesis may result in a lack of glucose and thus failure to meet the energy requirement of the brain, despite the abundantly available free thiamine present in the tissue. Elevated free thiamine in these affected brains might originate from thiamine treatment and from transfer of thiamine from tissues such as liver and muscle. Although thiamine treatment is thought to be account for much of the elevated free thiamine, this is not necessarily the case as thiamine is usually excreted from the body quickly as thiamine is a water soluble vitamin (Cebra and Cebra, 2004). However, a certain portion of free thiamine which accumulated in the PEM brains was likely transferred from thiamine storing tissues. It is also possible that the brain may require more thiamine because it could not get enough TPP.

Therefore, more thiamine may be continuously transferred from thiamine storing tissues to the brain. Elevated blood thiamine level observed in S-induced PEM affected animals (Gooneratne et al., 1989a; Olkowski et al., 1992) suggests this point.

The causes of insufficiency of TPP in brains of the 4 animals from the field could be due to the inhibition of TPP synthesis and/or enhanced TPP degradation. Thiamine pyrophosphate is synthesised from free thiamine by thiamine TPK (Liu and Hurley, 2011). This phosphorylation process is only accomplished under the conditions where there is enough free thiamine, ATP, Mg^{2+}, and normal function of TPK (Ramana et al., 2012). Hence the inhibition of TPP synthesis can occur when any one of the required conditions is altered. Free thiamine was abundant in the PEM brains and thus it can be ruled out as a factor inhibiting TPP synthesis. However, it is possible that alteration of ATP, Mg^{2+}, or TPK activity (Raghavendra Rao et al., 1993) inhibited TPP synthesis from free thiamine. Mastrogiacomo et al. (1996a) observed that TPP was significantly reduced while free thiamine and TMP remained unaltered in the brain of AD patients. Since ATP levels in the brains of AD patients are reduced, they proposed that TPP reduction was likely due to the reduction of TPK activity as TPK is an ATP dependent enzyme. Raghavendra Rao et al. (1993) also reported a 60% decrease in TPK activity in the brain of an AD patient that had decreased TPP (Heroux et al., 1996). Thiamine pyrophosphate synthesis cannot be performed when there is a lack of Mg^{2+}. This results in an apparent thiamine deficiency, even when the body has enough or excess thiamine (Johnson, 2001). Adverse effects of sulfite on mitochondrial ATP production have been reported (Zhang et al., 2004). Furthermore, the adverse effect of sulfate on Mg^{2+} absorption from the rumen is well documented (Ferreira et al., 1966a; Ferreira et al., 1966b; Martens and Blume, 1986). The adverse effects of S on these substances required for TPP synthesis may result in inhibition of

TPP synthesis. This is an area of focus for future study.

Enhanced degradation of TPP could be another factor attributing the reduced brain TPP in the PEM affected cattle. Sulfite, a strong nucleophile (Chiarani et al., 2008), may increase the degradation of TPP as TPP is a very active molecule and is readily degraded by strong nucleophiles (Belitz et al., 2009).

There are some weaknesses in the comparison of experimental heifers with field cases. It would have been more desirable if brain samples of clinically normal cattle from the same PEM affected feedlot were taken and compared with the clinical cases. However, as this was a field situation this was not practical. In addition, thiamine treatment of these clinical cases may have influenced brain thiamine metabolism. Collecting brain samples from natural cases of PEM without thiamine treatment is difficult due to 1) owners of feedlots always want to get suspected PEM cases treated immediately, 2) only those affected cattle that do not respond to thiamine treatment are available for brain collection. On the other hand, it would be also good if there had been clinical cases of PEM in the experimental animals. However, the experimental animals did not develop PEM even though they were exposed to excess dietary S. Given the fact that the incidence rate of PEM is low (often < 1 or 2%), it is challenging to induce PEM, particularly when a limited number of cattle are used. Considering these practical reasons, comparing experimental animals with field cases is reasonable.

5.6. Conclusion

To conclude, neither clinical nor histopathological signs indicative of PEM were detected in HS cattle despite an excess of S intake. There were no interactive effects of dietary S and F:C on thiamine metabolism. Dietary S did not influence ruminal and blood thiamine and its phosphate esters. Ruminal thiamine and its phosphate esters, as well as blood free thiamine were

increased by low F:C. Brains from the cattle fed HS diet exhibited increased TPP and total thiamine. Brain thiamine metabolism was not influenced by F:C. Dietary S increased free thiamine in the brain tissues of PEM animals while TPP levels were reduced. Brain thiamine data from experimental animals and field cases together suggest that dietary S may increase metabolic demand for TPP, and lack of TPP due to a defect in thiamine phosphorylation may lead to the development of malacic lesions in S-induced PEM affected animals.

6.0 EVALUATION OF SULFUR-MINERAL INTERACTIONS IN HIGH DIETARY SULFUR EXPOSED OR SULFUR-INDUCED POLIOENCEPHALOMALACIA AFFECTED BEEF CATTLE

6.1. Abstract

Sulfur-mineral interactions in heifers fed high S containing diets with differing F:C, and in S-induced PEM affected feedlot steers were evaluated. A metabolism trial was conducted as a randomized complete block using a 2 × 2 factorial treatment arrangement with main effects of dietary S and F:C using 16 ruminally cannulated heifers (initial BW 628 ± 48 kg). The F:C was modified by altering the proportion of barley silage (4 vs. 51% DM), whereas, the S content was modified by using differing sources of wheat DDGS (~38% DM) to achieve low and high S diets (LS = 0.30 vs. HS = 0.67% DM). Elemental S was also added to the pellets to increase the S content of HS diets. Mineral containing Cu, Co, Fe, Mg, Mn, Mo, Se and Zn from rumen fluid, blood, brain tissue and urine were analyzed. Daily urinary mineral excretion was also assessed. The mineral concentration from brains of 4 naturally occurring S-induced PEM affected steers were compared with the brains of HS heifers which were considered normal as they had no macroscopic or microscopic changes associated with PEM. There were no interactions between dietary S concentration and F:C ($P > 0.05$). Cattle fed HS diet showed reduced daily intakes of all minerals except for Mo ($P = 0.403$), which was caused by reduced feed intake. Daily intakes of Cu, Fe, Mo, and Se were greater ($P < 0.05$) with high F:C diet. Cattle fed HS diet had reduced Co ($P = 0.028$), Fe ($P < 0.001$) and Mn ($P < 0.001$) but similar ($P > 0.05$) Cu, Mg, Mo, Se and Zn concentrations in rumen fluid relative to those fed LS diet. Low F:C resulted in greater ruminal Cu ($P = 0.036$), Fe ($P< 0.001$), Mn ($P = 0.002$) and Zn ($P = 0.009$). Serum Mg ($P = 0.003$), Fe ($P = 0.036$) and Mn ($P = 0.100$) concentrations were reduced in HS cattle. Cattle fed

low F:C diet had reduced serum Cu (P = 0.027) and Se (P = 0.007) but greater Mg (P = 0.002) concentrations in comparison with those fed high F:C diet. High dietary S did not (P > 0.05) influence the brain minerals with the exception of reduced Se (P = 0.013). None of the minerals in the brain tissue was affected by F:C (P > 0.05). The PEM brains had reduced Cu (P = 0.058), Fe (P = 0.003) and Mo (P < 0.001) concentrations in comparison with normal brains. Other mineral concentrations did not differ among PEM and normal brains (P > 0.05). Results of the metabolic trial indicate that nutritional status of minerals such as Cu, Mo, and Zn associated with excess S intake was not detected. Mineral results in PEM brains suggest S interactions with Cu, Mo, or Fe may be implicated in the development of PEM lesions.

6.2. Introduction

Excessive S intake is associated with not only increased incidence of S-induced PEM but also reduced nutritional status in cattle. Excess S can interact with minerals such as Cu, Mo, Mg, Se, Fe and Zn, and influence the absorption and availability of these minerals. Copper deficiency is often seen in cattle fed high dietary S (Hill, 1969; Gooneratne et al., 1989a). High dietary S can reduce the bioavailability and absorption of Cu by forming CuS or more commonly, in combination with Mo through a Cu-thiomolybdate complex (Cammack et al., 2010; Gooneratne et al., 2011). Three-way interactions between S, Cu and Mo in ruminants have been recognized since 1956 (Dick, 1956). In addition, Cu status is also influenced by three-way interactions between Cu-S and Fe (Standish and Ammerman, 1971; Suttle and Field, 1983). High S may also influence Mg status in ruminants by reducing Mg absorption (Ferreira et al., 1966a; Ferreira et al., 1966b; Martens and Blume, 1986; Richter et al., 2012). Furthermore, high dietary S decreases Se bioavailability in cattle (Spears, 2003). Interaction between S and Se has

become evident since the observation of the link between white muscle disease in lambs and sulfate supplementation (Whanger et al., 1969). The adverse effect of high dietary S on Se status in ruminants was determined by several studies (White and Somers, 1977; Pope et al., 1979; Ivancic Jr. and Weiss, 2001). In addition, Van Ryssen et al. (1998) observed three-way interactions between S-Se-Cu in sheep. Based on limited information, high S may also interfere with Zn status in ruminants. High S increased urinary Zn excretion (Gooneratne et al., 2011) and reduced hepatic stores of Zn (Cammack et al., 2010).

Indeed, the adverse effect of high dietary S depends not only on the S concentration in feed and/or water, but also on the concentrations of the minerals that interact with S. The interactions between S and other minerals discussed above can reduce S toxicity by rendering the S biologically inactive. Hence considering the interactions between S and other minerals, it is important to understand the relationship between mineral metabolism and S toxicity in cattle fed.

Sulfur toxicity is influenced by composition of the diet. Therefore 2 different maximum tolerable levels of dietary S were set by the NRC, (2005); One for cattle fed forage (> 40% forage) based diets and one for concentrate (> 85% concentrate) based diets (0.30% vs. 0.50% S DM). Sulfide is the major form of S which interacts with other minerals. Sulfide in the rumen is produced through the reduction of inorganic S (sulfate and sulfite) and degradation of S containing amino acids entering the rumen (Lewis, 1954). It is primarily used for microbial amino acid (cysteine and methionine) synthesis (Emery et al., 1957). However, when excess sulfide is generated, it interacts with other minerals. Sulfide status in the rumen is influenced by dietary protein that is a source of S containing AA entering the rumen, as well as dietary carbohydrate content which greatly influences rumen pH. It is therefore possible that S and mineral interactions may be influenced by diet composition. Feeding DDGS which is rich in

minerals and protein but almost free from starch will create higher mineral and protein status and different rumen conditions compared to feeding typical feeds. To date, however, there is limited information regarding the S-mineral interactions in cattle fed DDGS based diets with differing F:C.

Furthermore, S-mineral interactions may be involved in the pathogenesis of PEM lesions in the brain. Three-way interactions between S, Cu and thiamine have been postulated to be involved in the development of malacic lesions (Olkowski et al., 1991; Gooneratne et al., 2011). As discussed in Chapter 5, a depressed TPP was detected in the brain of S-induced PEM affected cattle. One of the possible factors causing brain TPP depletion is the inhibition of TPP synthesis from free thiamine (Johnson, 2001). Inadequate Mg ions can inhibit TPP synthesis (Johnson, 2001). Considering the S-Mg interaction, it is possible that S may reduce Mg and thereby inhibit TPP production in the brain. In addition, Mo is reported to inhibit hepatic sulfide oxidation to sulfate (Ward, 1978). Since sulfide oxidation is the major detoxification process in tissue, it is plausible that the lower levels of Mo in the brain tissue may intensify S toxicity and thus lead to the development of malacic lesions. In the light of the interrelations between S and some minerals, it is important to evaluate mineral status of cattle fed high S diets that vary in F:C. Furthermore, mineral status in the brains of S-induced PEM affected animals has not been reported. The principle objective of the present study was to determine the effects of dietary S concentration and F:C on rumen, serum, brain and urinary mineral status in beef heifers. A secondary objective was to evaluate mineral status in the brains of S-induced PEM affected cattle.

6.3. Materials and Methods

Animal care, experimental design and treatments, as well as diet composition for metabolic trial were described in Chapter 4.

6.3.1. Sampling and Analysis

Feed and water sampling: Feed and water sampling from metabolic trial and field observation were described in Chapter 4 and 5, respectively.

Blood and rumen fluid sampling: Blood and rumen fluid samples were collected at 1200 on d 1, 7, 10, 14, 21, 28, and 35 of the experimental period. The sampling procedures were described in chapter 4. A 10 mL of rumen fluid sample and 6 mL blood sample which was collected with blue top vacutainer tube (BD Vacutainer®, Ref 368380) contained clot activator were immediately transferred to the laboratory for mineral analysis.

Urine sampling: Total urine was collected every sampling day (d 1, 7, 10, 14, 21, 28 and d 35) for determining urinary output and urinary mineral concentrations. Detail procedures of urine sampling were described in Chapter 4. Fifteen mL of urine was subsampled and immediately transferred to the lab for mineral analysis.

Brain tissue sampling: Brain mineral (Cu, Co, Fe, Mg, Mn, Mo, Se and Zn) status were evaluated from 16 experimental heifers and 4 PEM affected cattle. Brain sample collection procedures for both experimental and PEM affected animals were described in Chapter 5.

Mineral analysis: minerals such as Co, Cu, Fe, Mg, Mn, Mo, Se, and Zn from feed , water, rumen fluid, blood, brain and urine samples were analyzed using ICP – MS Spectrometer (Thermo Jarrel Ash – Corporation, Franklin MA) by Prairie Diagnostic Services Inc, Saskatoon, Canada.

History of clinical cases: For more detail information regarding the history of PEM cases the reader is referred to Chapter 5. The PEM affected cattle exhibited typical clinical signs as well as gross and microscopic changes in brain tissue consistent with PEM. Etiology of these field cases was determined as S-induced PEM based on gross and histopathological findings, as well as water and feed S content.

Feed and water sample analysis: Feed samples from metabolic trial were analyzed for CP, starch, ADF, NDF, Ca, P, Na, K and S by Cumberland Valley Analytical Services (CVAS, Hagerstown, MD). Sulfur concentration of feed samples collected from a PEM affected feedlot was analyzed by Agri-Food Laboratory of University of Guelph (Guelph, ON, Canada). Water sulfate was determined using Inductivity Coupled Plasma Atomic Emission Spectroscopy by Saskatchewan Research Council Analytical Laboratory (Saskatoon, Canada).

6.3.2. Statistical Analysis

Blood, rumen, urinary mineral data were analyzed as a randomized complete block (RCB) design with repeated measures with 2 × 2 factorial treatment arrangement using the Mixed Model Procedure of SAS (Version 9.2; SAS Institute Inc. Cary, NC). Since no significant block effect was observed, the block was removed from the model and data analyzed with the following model: fixed effects of F:C, S, F:C × S, day, F:C × day, S × day and F:C × S × day, and random effect of animal nested within the block. The covariance error structure for each model was chosen based on its lowest AIC value. Brain mineral data from experimental heifers were analyzed as RCB design with a 2 × 2 factorial treatment arrangement. After observing no significant block effect, the block was removed from the model and the final model included fixed effects of F:C, S and F:C × S, and random effect of animal nested within the block. The

Satterthwaite method was applied to determine degrees of freedom. Means were compared using Tukey's multiple comparison. Differences in minerals such as Cu, Co, Mn, Mo, Zn, and Se between PEM and HS brains were compared with 2-tailed t-test using the Mixed Model Procedure of SAS; while differences in Mg and Mn concentrations between PEM and HS brains were compared with Mann-Whitney test. Significance was declared at $P < 0.05$ and trends were discussed when $0.05 \leq P \leq 0.10$.

6.4. Results and Discussion

6.4.1. Sulfur and Mineral Intakes

Actual S concentration in the LS and HS diets averaged 0.31 and 0.62% (DM basis), respectively (Chapter 4, Table 4.1). The actual mean S concentration in HS diets was 0.05% lower than formulated value. This discrepancy resulted from slightly lower S content of the supplements to which elemental S was added during pelleting. Daily S intake was greater ($P < 0.01$) for heifers fed HS diets relative to those fed LS diets (53.0 vs. 34.5 g/d, respectively) but did not differ ($P = 0.53$) among cattle fed high F:C and low F:C diets (44.4 and 43.0g/d, respectively) (Chapter 4, Figure 4.3). Water S intake was not estimated, however sulfate content of the water used in this trial (110 mg/L) was low (Olkowski, 2009).

Data for daily mineral intakes are shown in Table 6.1. Mineral intakes only included intakes from the feed as water intake was not recorded. Mineral content of the water sample (Co: 3.3 µg/L, Cu: 77 µg/L, Fe: 87.8 µg/L, Mg: 16.4 mg/L, Mn: 12.5 µg/L, Mo: 1.55 µg/L, Se: 0.35 µg/L, and Zn: 108.2 µg/L) used for this trialwas low (Olkowski, 2009). Daily intakes of minerals for HS cattle were lower ($P < 0.01$) than LS cattle. The only exception was Mo intake ($P = 0.403$). Reduced mineral intake was a result of reduced DMI ($P < 0.001$) due to high dietary S (Chapter 4, Figure 4.3). Daily intake of Co, Mg, Mn and Zn did not vary ($P > 0.05$)

among cattle fed low F:C and high F:C diets. However, Cu, Fe, Mo, and Se intakes were greater ($P < 0.05$) in cattle fed high F:C diet than low F:C diet, likely due to the greater presence of these minerals in barley silage than barley grain (Table 6.3). Feed intake did not contribute to the increased intakes of these minerals with low F:C as there was no difference ($P = 0.261$) in DMI among the cattle fed the two F:C diets (Chapter 4, Figure 4.3).

Table 6.1. Effects of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on daily mineral intake from the feed

	Dietary treatments							
	F:C		Sulfur			*P*-value		
	Low	High	High	Low	SEM[z]	F:C	Sulfur	F:C × Sulfur
Co (mg/d)	3.23	3.11	2.50	3.84	0.127	0.533	< 0.001	0.157
Cu (g/d)	0.128	0.159	0.117	0.170	0.0057	0.002	< 0.001	0.068
Fe (g/d)	0.749	1.419	0.877	1.291	0.0442	< 0.0001	< 0.001	0.462
Mg (g/d)	20.50	22.02	19.22	23.30	0.812	0.206	0.003	0.640
Mn (g/d)	0.669	0.718	0.555	0.843	0.0278	0.234	< 0.001	0.182
Mo (mg/d)	9.78	14.72	11.97	12.53	0.461	< 0.0001	0.403	0.172
Se (mg/d)	8.83	11.73	9.45	11.11	0.393	0.0001	0.009	0.791
Zn (g/d)	1.17	1.25	1.10	1.32	0.046	0.247	0.005	0.551

[z]SEM = pooled standard error of the mean

Since concentrations of all minerals in all diets were higher than the recommended levels (NRC, 2000) (Table 6.2), overall intake of minerals in cattle fed either low or high F:C diet were expected to be higher than cattle fed non-DDGS based finishing or backgrounding diets. Higher concentrations of minerals particularly Cu, Mg, Se, Zn and Mn in treatment diets are likely due to the inclusion of DDGS. About 38% (DM) of wheat DDGS was included in all treatment diets (Chapter 4, Table 4.1). Mineral analysis of feed ingredients showed that average concentrations of Cu, Mg, Se, Zn, and Mn in high and low S containing DDGS were 105, 160, 233, 317, and 456% , respectively, higher than average concentrations of these minerals in barley grain and barley silage (Table 6.3). Such elevated mineral levels in DDGS are the result of starch removal

Table 6. 2. Mineral content of treatment diets (mg/kg DM basis ± SD)

Minerals	Treatment[z]			
	HGHS	HGLS	LGHS	LGLS
Co	0.30 ± 0.13	0.33 ± 0.10	0.28 ± 0.06	0.36 ± 0.04
Cu	12.22 ± 0.57	12.86 ± 0.39	14.99 ± 0.66	17.82 ± 0.87
Fe	62.97 ± 15.68	81.68 ± 3.28	143.8 ± 18.2	151.5 ± 11.8
Mg	2087 ± 18	1951 ± 123	2386 ± 4	2235 ± 118
Mn	61.02 ± 2.7	69.30 ± 2.94	65.55 ± 1.54	82.19 ± 3.32
Mo	1.12 ± 0.02	0.84 ± 0.03	1.68 ± 0.01	1.42 ± 0.06
Se	0.90 ± 0.10	0.84 ± 0.07	1.31 ± 0.20	1.16 ± 0.23
Zn	121.1 ± 28.3	110.9 ± 20.6	126.7 ± 29.0	126.5 ± 23.3

[z]Treatment: HGHS: low F:C high S; HGLS: low F:C low S; LGHS: high F:C high S; LGLS: high F:C low S

NRC (2000) recommended levels of minerals (mg/kg of DM) in growing and finishing cattle diets are: Co (0.1), Cu (10), Fe (50), Mg (1000), Mn (20), Mo (tolerable < 5), Se (0.1) and Zn (30)

from the source grain during the fermentation process at the ethanol plant (Batal and Dale, 2003). Overall mineral content of DDGS based diets are most likely to be higher than in typical grain or forage based diets.

Sulfur content of the feed offered to the cattle at the feedlot where field cases of PEM were investigated was 0.34% (DM). Sulfate concentration in water was 1755 ppm. Estimated daily S intake per head was 47 g/d, which was similar to the experimental heifers fed HS diets (P> 0.05). Other mineral content of the feedlot diet or water were not assessed.

6.4.2. Ruminal Mineral Status

No interaction (P > 0.05) between dietary S and F:C was observed for the mineral concentrations in the rumen fluid (Table 6.4). Ruminal concentrations of Co, Fe and Mn in HS cattle diet were reduced by 30, 42, and 28.5%, respectively, relative to LS cattle. However, ruminal Cu, Mg, Mo, Se and Zn concentrations did not vary (P > 0.05) among HS and LS cattle. Cattle fed the low F:C diet exhibited greater ruminal Cu (P = 0.036), Fe (P < 0.001), Mn (P = 0.002) and Zn (P = 0.009) compared to those fed the high F:C diet. Ruminal Co, Mg, Mo, and Se were not influenced by F:C (P > 0.05).

The reduced levels of Co, Fe and Mn in the rumen fluid of HS cattle was most likely due to the lower intakes of these minerals caused by dietary S rather than a direct antagonist effect of S on these minerals. Reduced ruminal Co in HS cattle may also be related to the indirect effect of S on Co. Cobalt is incorporated into vitamin B_{12} in the rumen by microorganisms (McDowell, 1992).Vitamin B_{12} synthesis is reported to be stimulated by S (Hunt et al., 1954). High dietary S may increase the rate of vitamin B_{12} synthesis with concomitant reduction of Co in the rumen as more Co is incorporated into B_{12}. Minerals are present in solid and liquid phases of the rumen

Table 6.3. Mineral concentrations (mg/kg, DM basis) of barley grain, barley silage and low (DDGS-NWT) and high (DDGS-TG) sulfur containing dried distillers grains with solubles (DDGS)

	DDGS-NWT (n=3)	DDGS-TG (n=3)	Barley grain (n=4)	Barley silage (n=4)
Co	0.14	0.07	0.36	0.24
Cu	9.23	10.37	4.24	5.32
Fe	69.30	65.80	44.35	176.30
Mg	3234	3592	1056	1575
Mn	116.33	98.80	15.03	23.48
Mo	1.08	1.83	0.63	1.82
Se	1.13	1.67	0.17	0.67
Zn	157.63	199.67	43.15	38.65

Table 6.4. The effect of dietary sulfur concentration and forage-to-concentrate (F:C) on rumen, serum and brain mineral status in beef heifers

	Dietary treatments				SEM[z]	P-value		
	F:C		Sulfur					
	Low	High	High	Low		F:C	Sulfur	F:C × Sulfur
Rumen minerals								
Co (mg/L)	3.812	3.016	2.811	4.018	0.3702	0.138	0.028	0.526
Cu (mg/L)	0.42	0.135	0.185	0.37	0.0738	0.035	0.128	0.192
Fe (mg/L)	2.229	1.322	1.302	2.25	0.1469	<0.001	<0.001	0.100
Mg (mg/L)	162.08	165.11	158.62	168.57	7.315	0.772	0.346	0.550
Mn (mg/L)	4.182	3.248	3.097	4.332	0.207	0.002	<0.001	0.370
Mo (mg/L)	0.023	0.016	0.018	0.022	0.0036	0.177	0.432	0.444
Se (mg/L)	0.030	0.031	0.032	0.029	0.0024	0.806	0.282	0.236
Zn (mg/L)	1.134	0.639	0.875	1.112	0.1736	0.009	0.346	0.540
Serum minerals								
Co (µg/L)	0.393	0.320	0.406	0.419	0.0317	0.412	0.775	0.387
Cu (mg/L)	0.697	0.780	0.741	0.735	0.0246	0.027	0.855	0.898
Fe (mg/L)	1.077	1.146	1.003	1.220	0.0698	0.487	0.036	0.382
Mg (mg/L)	21.50	19.34	19.38	21.46	0.475	0.002	0.003	0.279
Mn (mg/L)	0.011	0.014	0.005	0.019	0.0056	0.733	0.100	0.699
Mo (mg/L)	0.013	0.011	0.012	0.013	0.0028	0.647	0.700	0.799
Se (mg/L)	0.174	0.189	0.184	0.179	0.0039	0.007	0.322	0.517
Zn (mg/L)	0.89	0.848	0.903	0.835	0.0391	0.453	0.224	0.008
Brain minerals								
Co (µg/g, wt)	0.014	0.014	0.015	0.014	0.0030	0.974	0.721	0.423
Cu (µg/g, wt)	4.32	4.16	4.22	4.27	0.347	0.746	0.921	0.644
Fe (µg/g, wt)	25.49	25.05	25.93	24.61	1.674	0.857	0.59	0.662
Mg (µg/g, wt)	142.18	144.35	147.23	139.30	6.590	0.819	0.411	0.743
Mn (µg/g, wt)	0.33	0.484	0.516	0.298	0.1317	0.425	0.263	0.331
Mo (µg/g, wt)	0.051	0.054	0.054	0.051	0.0026	0.374	0.446	0.815
Se (µg/g, wt)	0.323	0.336	0.351	0.308	0.0106	0.378	0.013	0.807
Zn (µg/g, wt)	12.86	12.50	13.00	12.36	0.377	0.509	0.255	0.801

[z]SEM = pooled standard error of the mean

contents. The majority are present in the solid phase due to their presence in plant material and adherent micro-organisms (Durand and Kawashima, 1980; Allen and Gawthorne, 1987). The proportion of a mineral present in solid or liquid phases may be influenced by the levels of its antagonists in the rumen content. The addition of an antagonist may lead to an increase in the level of affected mineral in the solid phase at the expense of its concentration in the liquid phase (Allen and Gawthorne, 1987). It is possible that the reduced Fe concentration in the rumen fluid of HS cattle would partially have resulted from the antagonist effect of S. Surprisingly, Cu, Mg, Se, and Zn concentrations in the rumen fluid did not differ among cattle fed HS and LS diets regardless of the decreased intake of these minerals in HS cattle. The reason for this observation is not clear.

Despite lowered intake, greater Cu and Fe concentrations were observed in the rumen fluid of cattle fed low F:C diet. Also elevated Mn and Zn concentrations in the rumen fluid of cattle fed low F:C were detected despite similar intakes of these minerals between low and high F:C diets. In contrast, Se, Mg and Mo intakes were greater with the high F:C diet, but ruminal concentrations of these minerals were not different between low and high F:C diets. Taken together this data indicates that the availability of minerals in the rumen from grain-based diets may be higher than that of forage-based diets. This may be due to differences in: 1) the rumen environment (rumen volume, pH) created by the different dietary F:C, 2) the release of minerals from the grain and forage into the rumen, and 3) the rate of fermentation of grain and forage in the rumen (Johnson and Aubrey Jones, 1989).

6.4.3. Serum Mineral Status

There was no interaction ($P < 0.05$) between dietary S concentration and F:C for serum

mineral concentrations except for Zn (P = 0.008) (Table 6.4). Serum Mg (P = 0.003) and Fe (P = 0.036) were reduced in heifers fed the HS diet. Serum Mn concentrations tended (P = 0.10) to be reduced by the HS diet. However, the rest of the serum minerals were not influenced by the HS diet (P > 0.05). Cattle fed low F:C diet had lower serum Cu (P = 0.027) and Se (P = 0.007) but higher Mg (P = 0.002) concentrations in comparison with those fed high F:C diet. The remaining minerals in the serum were not affected by F:C (P > 0.05).

Although ruminal Mg concentrations between HS and LS cattle were not different, reduced serum Mg concentrations in HS cattle were detected. Similar to our results, Spears et al., (1985) observed a reduced availability and absorption of Mg in steers fed forages higher in S. Richter et al. (2012) also reported that plasma Mg concentration of backgrounding steers was decreased as dietary S increased. Reduced serum Mg in HS cattle could be owing to the inhibitory effect of high S on absorption of Mg across the rumen wall and/or formation of an insoluble complex of S and Mg in the rumen. In vitro and in vivo studies showed that substitution of Cl^- with SO_4 ions results in an increased transmural potential difference which reduces Mg absorption (Ferreira et al., 1966a; Ferreira et al., 1966b). In addition, Martens and Blume (1986) reported that in vitro, sulfate is a more potent inhibitor of Mg uptake across the rumen epithelium than Cl^- due to a rise in lumen pH. It is also possible that S and Mg interact in the rumen and form an insoluble complex (Richter et al., 2012).

Reduced serum Fe in cattle fed HS diet most likely was due to the lower ruminal Fe concentration in HS cattle resulting from reduced intake of Fe. However, it is still possible that the ruminal interaction between Fe and S may partially account for the decrease in serum Fe in HS cattle. Iron in the rumen can interact with sulfide and form ferrous sulfide (Suttle et al., 1984).

Since the possibility of an interaction between S and Mn in ruminants has not been identified, reduced serum Mn in HS cattle is more likely due to the lower ruminal Mn level.

Unaltered serum Cu and Mo status combined together with unaltered ruminal Cu and Mo status in HS cattle indicates that there were no interactions between Cu, Mo or S in cattle fed HS diet. High dietary S can reduce the bioavailability and absorption of Cu either alone by forming CuS (Suttle, 1974b; Cammack et al., 2010) or more commonly in combination with Mo through forming a Cu-thiomolybdate complex (Dick, 1956; Suttle and Field, 1983; Gooneratne et al., 1989a; Suttle, 1991; Cammack et al., 2010). The fact that serum Cu concentration was not altered by high dietary S (0.62 % DM) in the present study could be related to characteristics of S, Cu and Mo presenting in DDGS as well as the ruminal environment created by the DDGS which is a low starch feedstock.

High dietary S can reduce the bioavailability of Se in ruminants (Spears, 2003). Adverse affects of dietary S on Se availability and absorption were detected in several studies (Whanger et al., 1969; White and Somers, 1977; Pope et al., 1979). Three-way interactions between Cu, Se and S were also identified (Van Ryssen et al., 1998). However, in the present study, serum Se was not influenced by high dietary S. This indicates that there were neither interaction between S and Se in the rumen nor was there an inhibitory effect of S on Se absorption. The reason for not seeing this interaction may again be related to the form of the S and amount of mineral in DDGS and the rumen environment created by this starch free feedstock.

When excessive S is ingested, Zn can interact with S and form a ZnS complex. Precipitation of the ZnS complex reduces Zn absorption (Qi et al., 1993). However, excess S intake did not influence Zn absorption in the present study. There is no apparent explanation for why serum Co was not influenced by the HS diet as it was reduced by HS diet in the rumen.

Despite of the greater presence of Cu in the rumen, reduced serum Cu was detected in cattle fed low F:C diet relative to cattle fed high F:C diet. This may related to the fact that Cu in the rumen of cattle fed grain based diet may be less available to the animal or the absorption of this mineral might be inhibited by acidic rumen conditions caused by high grain diet. Copper can be absorbed from the rumen (McDowell, 1992). When cattle are fed high grain diets, it is possible that the epithelial barrier function may become more restrictive (Lodemann and Martens, 2006) and prevent Cu absorption across the rumen wall thereby reducing the concentration of serum Cu.

Reduced serum Se was detected in cattle fed low F:C diet regardless of ruminal Se levels between cattle fed low or high F:C diets. This indicates that Se in the grain-based diet was less available. This result however contrasts with other research where Se has been reported to be more available to animals on grain based diets than forage based diets (Spears, 2003). Koenig et al. (1997) observed that Se availability was greater in sheep fed high grain diet (77.9% barley grain) relative to those fed a forage-based (77% alfalfa hay) diet. The discrepancy between our findings and others in terms of Se availability might be related to the type of the forage included in these studies. As Se is not absorbed from the rumen (McDowell, 1992), changes in ruminal epithelial barrier function are not considered to be a reason to account for reduced Se absorption in low F:C cattle.

Elevated serum Mg concentration in cattle fed the low F:C diet indicates high grain diet may provide greater available Mg than forage based diets. Richter et al. (2012) observed that plasma Mg was reduced by high dietary S in cattle fed a backgrounding diet but not affected by high dietary S in those fed a finishing diet. They postulated that the reduced plasma Mg concentration in backgrounding cattle might be due to favorable rumen conditions for forming S and Mg complex. However, the results of the present study indicated that Mg might be lower in

cattle fed backgrounding diets and therefore the Mg in these cattle might be more vulnerable to S effects relative to cattle fed a finishing diet.

6.4.4. Brain Mineral Status

Experimental cattle: No interaction between dietary S concentration and F:C was observed for brain mineral concentrations ($P > 0.05$). High dietary S did not ($P > 0.05$) influence the brain mineral concentration with the exception of Se ($P = 0.013$). None of the minerals in the brain tissue were affected by F:C ($P > 0.05$) (Table 6.4).

Mineral concentrations from S-induced PEM affected brains were compared with that of experimental cattle fed HS diet. Brains of HS cattle were chosen to compare with PEM brain as normal brain as HS cattle had similar S intake to the PEM cattle. They were considered as "normal"as they did not have any gross or microscopic changes indicative of PEM. The PEM brains had reduced Cu ($P = 0.058$), Fe ($P = 0.003$) and Mo ($P < 0.001$) concentrations in comparison with normal brain. Other mineral concentrations did not differ among PEM and normal brains ($P > 0.05$) (Table 6.5).

Selenium concentration in the brain tissues of experimental cattle was reduced by high dietary S. The mechanism of S and Se interaction is not understood, however, the low content of Se might be related to its similar chemical properties of S (White et al., 2007) which may interfere with Se metabolism in the brain and thus enhance excretion of Se. Animals with decreased brain Se exhibited increased urinary Se concentrations (Table 6.5). Selenium and Se-dependent enzymes are important modulators of brain function. Selenium deficiency is reported to be associated with increased neuronal cell loss in models of neurodegenerative disease (Schweizer et al., 2004). However, depletion of brain Se does not seem to be implicated in the

development of brain PEM lesions, since all brains of HS cattle were free from any gross or histopathological lesions (Chapter 5). In addition, the unaltered Se status in the brain of PEM affected cattle (Table 6.5) also indicates that Se depletion was not involved in the development of malacic lesions. However, it is unclear why the depletion of Se in PEM affected cattle was not observed as the S intakes of these cattle were similar to that of experimental animals fed HS diet.

Table 6.5. Comparison of brain mineral concentrations (Mean ± SE) between normal and sulfur- induced PEM affected beef cattle

Minerals (µg/g, wet wt)	Normal brain (n = 8)	PEM brain (n = 4)	*P*-value
Co	0.015 ± 0.003	0.012 ± 0.003	0.519
Cu	4.22 ± 0.0.34	2.94 ± 0.51	0.058
Fe	25.93 ± 1.83	15.45 ± 1.03	0.003
Mg	147.2 ± 5.76	135.1 ± 2.30	0.293
Mn	0.516 ± 0.184	0.298 ± 0.017	0.199
Mo	0.054 ± 0.002	0.037 ± 0.003	< 0.001
Se	0.351 ± 0.01	0.365 ± 0.04	0.746
Zn	13.00 ± 0.43	12.93 ± 0.54	0.920

Interactions between Cu, Mo, and S are believed to occur not only in the rumen but also in systemic tissues (Suttle, 1991; Qi et al., 1993). Such interactions were detected in the brain tissues of PEM affected cattle while not seen in the experimental cattle. The reasons for not seeing the interactions between Cu, Mo and S in experimental animals may relate to the characteristic of S, Cu and Mo present in DDGS. Sulfur in DDGS seems to be less toxic than other S derived from feed, water or other sources (Chapter 4). This can be seen from the results of Amat et al. (2012) where cattle fed DDGS (0.51% or 0.65% S, DM) did not develop S-induced PEM. Similar results in sheep and cattle were reported by Neville et al. (2010); and

Neville et al. (2012).

Reduced Cu, Mo and Fe in the PEM brain tissue seems to be caused by S through three-way interactions between Cu-Mo-S, Cu-Fe-S or Fe-S. Although no rumen and blood mineral assessments were performed from these affected PEM cattle, interactions between S and these minerals likely occurred in the rumen and systemically.

Sulfur reacts with Mo to form different classes of thiomolybdates such as tetrathiomolybdate, dithiomolybdate and trithiomolybdate in the rumen. Tetrathiomolybdate can react with Cu and form a complex that is poorly absorbed (Qi et al., 1993); whereas, dithiomolybdate and trithiomolybdate can be absorbed and interact with Cu in the tissues so as to influence systematic Cu metabolism (Suttle, 1991). In addition, high dietary S, independent of Mo, can also reduce Cu in the rumen and tissues by forming insoluble CuS complex (Cammack et al., 2010). As well, Cu and Fe may be reduced by the interaction between Cu-Fe-S (Standish and Ammerman, 1971; Suttle et al., 1984). Iron in the rumen is postulated to react with sulfide and form ferrous sulfide complex that may become soluble in the abomasum where the sulfide may dissociate and form insoluble complexes with Cu (Suttle et al., 1984). Furthermore, the antagonist effect of Fe on Cu may also be involved in the reduction of these minerals in PEM cattle. Suttle (1991) postulated that Fe_20_3can bind Cu in the rumen.

Interactions between S and other minerals such as Cu not only reduce the absorption and bioavailability of the affected minerals, but also reduce S availability and absorption, thereby reducing its toxicity. Reduced Cu in PEM brains may allow for higher sulfide in the brain tissue that could exert its toxic effects on neuronal cells thereby enhancing the development of malacic lesions. Copper supplementation is reported to reduce the incidence of S-induced PEM, possibly through its protective function on thiamine metabolism from sulfide or sulfite toxicity (Olkowski

et al., 1991; Olkowski, 1997). The PEM affected brains have been shown to exhibit TPP depletion, although the finding was based on limited data set (Chapter 5). It is possible that there may be a link between reduced Cu and depletion of TPP in the affected brains. Depletion of brain Cu may result in greater amount of sulfide or sulfite ions in the brain tissue that would eventually exert its adverse effect on thiamine metabolism and thereby cause depletion of brain energy metabolism.

Since molybdenum is a cofactor for sulfite oxidase that regulates conversion of sulfite into sulfate (Cramer et al., 1979; Cramer et al., 1981), depletion of Mo in the brain may inhibit sulfide oxidation to sulfate with concomitant increases of both sulfide and sulfite ions in the brain. Sulfide is regarded as a major form of S causing PEM lesions by inhibiting cytochrome c oxidase resulting in depletion of brain energy metabolism (Olkowski, 1997; Gould, 1998). However, sulfite is also postulated to be involved in the development of malacic lesions (Olkowski, 1997). Sulfite, a strong nucleophile, can cleave thiamine and is also involved in degradation of TPP (Belitz et al., 2009). It is also possible that the adverse effects of sulfite on mitochondrial ATP production (Zhang et al., 2004) might indirectly inhibit TPP synthesis from free thiamine, as TPP synthesise requires ATP. It is plausible that depletion of Mo may also be involved in the development of neurotic lesions by increasing sulfite ions that may enhance TPP depletion in the brain or may exert its toxicity on the neuronal cells.

Iron can also alleviate S toxicity by forming FeS complex (Suttle et al., 1984). Depletion of Fe in the brain may also intensify S toxicity. Thus, attention should also be given to possible involvement of interactions between S and Cu, Mo and Fe in pathogenic development of S-induced PEM lesions.

6.4.5. Urine Mineral Status

There was no ($P > 0.05$) interaction between dietary S concentration and F:C for urine mineral concentrations except for Cu ($P = 0.04$). However, with respect to daily urinary mineral excretion there was interaction between dietary S and F:C Cu ($P = 0.03$) and Fe ($P = 0.035$). Concentrations of minerals in the urine did not vary ($P> 0.05$) among the cattle fed HS and LS diets with an exception of Se. Cattle fed HS diet had increased urinary Se concentration ($P = 0.047$) relative to those fed LS diet (Table 6.6).

Reduced daily urinary excretions of Fe ($P = 0.005$), Mo ($P = 0.01$) and Zn ($P = 0.031$) in HS cattle was observed. This might be due to reduced urinary output by high dietary S since no alterations of these mineral concentrations were seen in the urine. Urinary Co ($P = 0.077$) and Mg ($P < 0.001$) concentrations increase in cattle fed low F:C diet. Cattle fed low F:C diet had increased daily urinary excretions of Co ($P = 0.035$) and Mg ($P < 0.001$), but decreased daily urinary excretions of Cu ($P = 0.026$) and Se ($P = 0.046$) relative to those fed high F:C diet. The increase in Mg excretion might be related to the enhanced Mg absorption in cattle fed low F:C diet.

6.5. Conclusion

Most commonly reported interactions between S-Cu-Mo or S-Cu-Fe, or S-Zn were not observed in experimental cattle regardless of the excess S intake. High dietary S reduced Mg availability and absorption. Forage-to-concentrate ratio of the diet may influence the availability and absorption of Cu, Se, and Mg. Sulfur induced PEM affected cattle exhibited lower brain Cu, Mo and Fe relative to clinically normal cattle, suggesting the possibility of interactions between S and these minerals in the brain tissues.

Table 6.6. The effect of dietary sulfur concentration and forage-to-concentrate ratio (F:C) on urinary mineral status in beef heifers

	Dietary treatments							
	F:C		Sulfur			*P*-value		
	Low	High	High	Low	SEM[z]	F:C	Sulfur	F:C × Sulfur
Co concentration (µg/L)	1.24	0.80	1.15	0.90	0.162	0.077	0.303	0.893
Co output (µg/d)	13.87	11.17	12.58	12.46	0.815	0.035	0.916	0.068
Cu concentration (µg/L)	26.67	28.58	30.39	24.86	2.650	0.615	0.152	0.040
Cu output (µg/d)	304.3	395.1	352.4	347.0	25.54	0.026	0.885	0.003
Fe concentration (µg/L)	155.5	111.4	141.1	125.8	33.59	0.368	0.752	0.718
Fe output (µg/d)	1799.2	1564.3	1393.2	1970.2	121.97	0.196	0.005	0.035
Mg concentration (mg/L)	327.2	140.5	247.0	220.7	15.91	< 0.001	0.247	0.270
Mg output (g/d)	3.65	1.98	2.60	3.03	0.177	< 0.001	0.104	0.449
Mn concentration (mg/L)	2.83	2.71	3.20	2.34	0.382	0.828	0.114	0.588
Mn output (mg/d)	33.07	37.55	37.91	32.71	2.470	0.221	0.160	0.106
Mo concentration (mg/L)	0.15	0.15	0.14	0.16	0.015	0.682	0.433	0.439
Mo output (mg/d)	1.78	2.04	1.61	2.22	0.143	0.214	0.010	0.126
Se concentration (mg/L)	0.36	0.36	0.39	0.33	0.021	0.978	0.047	0.236
Se output (mg/d)	3.96	5.01	4.38	4.58	0.335	0.046	0.682	0.911
Zn concentration (µg/L)	183.07	146.41	163.75	165.73	39.650	0.527	0.973	0.927
Zn output (µg/d)	2069.5	2059.9	1811.9	2317.5	148.61	0.964	0.031	0.535

[z]SEM = pooled standard error of the mean

7.0 GENERAL DISCUSSION, SUGGESTION FOR FUTURE RESEARCH, AND GENERAL CONCLUSION

7.1. General Discussion

This thesis contained data from a small pen study, a metabolism study and a field observation. The effects of feeding DDGS from corn, wheat or a 50:50 corn:wheat blend DDGS on serum sulfate levels of feedlot steers were evaluated in small pen study. Sulfur and thiamine metabolism, mineral status, as well as SCFA absorption in cattle fed high S containing DDGS based diet with differening F:C ratio were evaluated in the metabolic trial. Thiamine and mineral status in the brains of S induced PEM affected cattle were also evaluated.

7.1.1. The Effect of Feeding Different Types of DDGS, and Dietary Sulfur Concentration and F:C on Serum Sulfate Levels of Beef Cattle

Since sulfate is the end product of sulfide entering the tissue, it is possible that measuring serum sulfate may be a means to identify cattle at risk of PEM. Serum sulfate results from the small pen study and metabolism trial together demonstrated that serum sulfate reflect dietary S intake in DDGS fed cattle. These results are in agreement with Weir and Rendig (1954) and Hansard and Mohammed (1969), who observed that serum sulfate reflected total S intake in sheep and cattle, respectively.

An interesting observation from thesmall pen study was that serum sulfate levels for cattle fed CDDGS and WDDGS were significantly higher during backgrounding than finishing phase despite the lower S intake in the backgrounding phase. Thus, it was hypothesized that high grain in the finishing diet may attribute to the low serum sulfate concentration in finishing

steers. It was also suggested by Neville et al., (2010) that there may be interactive effects of dietary S and dietary grain supplementation in finishing rations. However, this hypothesis was not supported by the results from the metabolic trial in which serum sulfate levels in heifers were not influenced by the F:C. It can be concluded that the lower serum sulfate levels in finishing steers may be related to other factors rather than the grain level of the finishing diet. Serum sulfate was positively correlated with the ruminal H_2S in the metabolic trial. This indicates that serum sulfate could be an indicator for identifying the risk of PEM according to current dogma that H_2S is the major factor for S-induced PEM. Since no single animal developed PEM in either the small pen study or the metabolic trial, correlating serum sulfate level with S-induced PEM was not accomplished.

7.1.2. The Effect of Dietary Sulfur Concentration and Forage-to-Concentrate Ratio on Dry Matter Intake and Ruminal pH

The metabolic trial results showed that high dietary S reduced DMI in heifers. The negative impact of high dietary S on feed intake in cattle has been reported by several studies (Sarturi et al., 2011; Uwituze et al., 2011a; Uwituze et al., 2011b). One of the possible reasons for the reduced DMI in cattle fed HS diet might be due to suppressed ruminal motility resulting from increased ruminal H_2S production. Reduced DMI in cattle fed HS could also partially be related to the difference in S content of two different wheat DDGS included in the treatment diets. No significant effect of F:C on DMI was observed.

High dietary S increased ruminal pH. Similar results have been reported by Uwituze et al. (2011a), who reported that high dietary S increased ruminal pH in steers. Since the cattle fed HS diet had reduced feed intake, elevation of rumen pH in cattle fed HS most likely resulted from

reduced DMI as feed intake is a primary factor influencing ruminal pH (Nordlund, 2003). As expected, low F:C diet reduced mean, minimum and maximum rumen pH, however, the reduction was not severe. The mean rumen pH in all treatments was above 6.0. Only heifers fed low F:C and LS diets experienced (2.7 and 2.3 h per day, respectively) significant time where rumen pH was under 5.5. Heifers fed high F:C and HS diets did not experience rumen acidosis.

7.1.3. The Effects of Dietary Sulfur Concentration and Forage-to-Concentrate Ratio on Sulfur Metabolism

Cattle fed HS diet had significantly greater ruminal H_2S concentration than those fed LS diet, reflecting S intake. Although cattle fed the HS diet had greater ruminal H_2S level than the suggestive toxic level (2000 ppm), these cattle did not show any clinical signs, nor any macroscopic, or microscopic changes in the brains indicative of PEM. Ruminal H_2S concentration was not influenced by F:C. Feeding a high grain diet has been considered to create a lower rumen pH that favours H_2S generation and thereby increase the incidence of S-induced PEM (Gould, 1998). Heifers fed the low F:C diets had lower rumen pH but only differed in mean pH by 0.28 units from rumen pH in cattle fed high F:C. This pH difference may not be big enough to produce different ruminal H_2S concentrations among these two diet types.

High S diets resulted in greater ruminal S^{2-} concentration relative to LS diets. It has been well documented that ruminal S^{2-} positively reflects S intake due to the fact that all S compounds in the rumen are converted into ruminal S^{2-}(Lewis, 1954). Unlike the other S metabolites evaluated in this study, ruminal S^{2-} was increased by high grain diet. This may be due to reduced rumen pH which provides a more favourable condition for sulfate reducing bacteria to produce

more sulfides in the rumen (Gould, 1998). Unfortunately, overall ruminal concentrations were significantly lower than the reported deficient level. This was most likely caused by the time between collection and analysis of S^{2-} in the present study. It has to be stressed that making any conclusion based on the ruminal S^{2-} data in the present study is difficult.

Although high dietary S tended to reduce urinary output, urinary sulfate excretion was still greater for cattle fed HS diet than those fed LS due to the remarkable increase in sulfate concentration in the urine. The results of the urine sulfate excretion further supported the proposed mechanism that increased sulfate excretion would be one of the possible ways for cattle exposed to high dietary S to avoid S toxicity (Cozannet et al., 2010; Neville et al., 2011).

7.1.4. The Effect of Dietary Sulfur Concentration and Forage-to-Concentrate Ratio on Short Chain Fatty Acid Absorption

One novel finding from the present study was that high dietary S inhibited SCFA absorption from the rumen. Reduced absorption of SCFA from the rumen of cattle fed HS diet could be due to the inhibited SCFA metabolism in ruminal epithelium. Sulfides are reported to inhibit butyrate and acetate oxidation in colonic epithelial cells of humans and rats (Roediger et al., 1993a; Roediger et al., 1993b; Leschelle et al., 2005). Since SCFA oxidation in epithelial cells is inhibited by sulfides, it is possible that the reduced absorption of SCFA from the rumen would occur due to inhibiting oxidation of SCFA in rumen epithelium as epithelial SCFA metabolism influences the absorption of SCFA. This finding may help to explain why reduced performance is often seen in animals exposed to high dietary S. It is interesting to see whether the inhibitory effect of S on SCFA absorption have implications for S toxicity as impaired absorption of SCFA and/or epithelial metabolism of the acids may result in destruction of

epithelial barrier function, which may allow more sulfides passing through the rumen epithelial.

Low F:C diets increased SCFA absorption rate by more than 50% compared to high F:C diets. This might be due to the stimulatory effect of grain on absorption of SCFA by increasing the absorptive surface of ruminal epithelium.

7.1.5. The Effect of Dietary Sulfur Concentration and Forage-to-Concentrate Ratio on Thiamine Metabolism

The results of metabolic trial showed that neither ruminal nor blood thiamine and its phosphate esters were influenced by high dietary S. Detrimental effects of high dietary S on ruminal thiamine and blood thiamine has been reported by several studies. Cattle affected by S-induced PEM were found to have insufficient blood thiamine (Gooneratne et al., 1989b). Unlike these reports, the reason for not seeing the detrimental effect of S on ruminal and blood thiamine status might be related to the inclusion of DDGS in the diets.

Brain TPP, TMP, and total thiamine in cattle fed HS diet were increased, indicating that animals exposed to high dietary S may have higher metabolic demand for thiamine. Data from S-induced PEM affected cattle showed that there was a significant reduction of TPP in PEM brains regardless of the elevated free thiamine. This suggests that these affected animals were unlikely be able to meet their demand for TPP. Considering the brain thiamine data from experimental cattle and S-induced PEM affected cattle, it can be speculated that there may be a continuous increase in the demand for TPP as S intake increases. Once the increase in TPP demand reaches a certain point, the host may not be able to maintain enough supply of TPP to meet demand. This may subsequently lead to the development of PEM lesions.

Thiamine data from field cases also sheds light on a possible explanation for the lack of response from some PEM affected cattle to thiamine treatment. Most animals responded well to thiamine treatment (Harries, 1987; Beke and Harionaka, 1991); while some animals like the field cases presented did not respond thiamine treatment (Jeffrey et al., 1994; Bulgin et al., 1996). The lack of response to thiamine treatment has been proposed to be due to the fact that brain lesions were advanced beyond clinical recovery (Olkowski, 1997). Thiamine status in PEM brains however, indicated that the lack of thiamine treatment response may be owing to metabolic weakness in converting free thiamine into TPP, which can be seen from the elevated free thiamine but reduced TPP concentrations in those PEM brains. The causes of insufficiency of TPP in brains of those field cases could be due to the inhibition of TPP synthesis and/or enhanced TPP degradation.

Ruminal thiamine and its phosphate esters were greater in cattle fed low F:C. Serum and brain thiamine status was not influenced by the F:C.

7.1.6. The Effect of Dietary Sulfur Concentration and Forage-to-Concentrate Ratio on Mineral Status

The mineral content of the experimental diets suggest that overall mineral contents of DDGS based diets are most likely higher than that of typical grain or forage based diets.

Unaltered ruminal and blood Cu and Mo status combined with unaltered brain Cu and Mo status in HS cattle indicates that there were no interaction between Cu, Mo or S in cattle fed HS. High dietary S can reduce the bioavailability and absorption of Cu either alone by forming CuS or more commonly in combination with Mo through forming a Cu-thiomolybdate complex.

Interactions between Cu, Mo, and S are believed to occur not only in the rumen but also in systemic tissues. The reasons for the interactions between Cu, Mo and S not occurring in experimental animals are not clear but most likely related to the forms of S, and amount of Cu and Mo presenting in DDGS.

Brain mineral status in experimental heifers was not influenced by high dietary S except for Se. Comparison between mineral status from PEM and normal brains revealed that PEM brains had reduced Cu, Fe and Mo concentrations. Reduced Cu, Mo and Fe in PEM brains are most likely to be caused by S through three-way interactions between Cu-Mo-S, Cu-Fe-S or Fe-S. Interactions between S and other minerals not only reduce the absorption and bioavailability of the minerals that interact with S, but also reduce S availability and absorption, thereby reducing its toxicity. Reduced Cu in the PEM brains may enable higher sulfide presenting in the brain tissue that could exert its toxicity on neuronal cells thereby enhancing the development of malacic lesions.

Availability of minerals such as Cu, Fe, Mg, Mn, Mo, Se and Zn in the rumen from grain based diets may be higher than that of forage based diets. Forage to concentrate ratio of the diet may influence the availability and absorption of Cu, Se, and Mg. Brain mineral status of experimental heifers was not influenced by the F:C.

7.1.7. Overall Evaluation of Sulfur Toxicity in Dried Distillers Grains with Solubles and the Effects of F:C Ratio on Sulfur Toxicity

Sulfur concentration in DDGS based diets frequently exceeds the maximum recommended level of 0.4% due to the high S content in DDGS. Therefore, feeding DDGS is

believed to have the potential to induce PEM in cattle. However, in the small pen study, neither the steers fed 40% CDDGS with 0.51% S (DM) or those fed WDDGS with 0.65% S developed PEM. Likewise, in the metabolic study, the heifers fed 0.62% S derived mainly from WDDGS (~38%) on either low or high F:C diets didn't show clinical signs of PEM, or any gross and microscopic changes indicative of PEM in their brains. Similar to the present studies, steers fed 30% CDDGS with 0.65% did not develop any clinical signs of PEM (Uwituze et al., 2011a; Uwituze et al., 2011b). In addition, lambs receiving finishing diets containing 60% DDGS with either 0.73% or 0.87% S did not develop any clinical PEM lesions. Furthermore, adverse effects of S on thiamine and mineral (Cu, Mo, Fe, and Zn) status were not observed despite the excess S intake. All together, this data suggest that the apparent toxicity of S presenting in DDGS seems to be lower than other forms of S. Therefore, the maximum tolerable level of S for cattle fed DDGS based diet should be reconsidered.

Forage based diets, as many other researchers have claimed, can reduce the incidence of S induced PEM. While concentrate based diet may intensify the toxicity of S and increase the PEM incidence. However, no differences in ruminal H_2S, serum sulfate as well as urinary sulfate in cattle fed low F:C and high F:C diets indicated that S toxicity may not be influenced by F:C ratio.

7.2. Suggestions for Future Research

1) To establish serum sulfate as a means to identify cattle with the risk of PEM, further research is required to correlate serum sulfate level with S-induced PEM.

2) Further studies are needed to elucidate the mechanism of the inhibitory effect of dietary S on SCFA absorption as well as the effect of dietary S on rumen epithelial barrier function.

3) To confirm the involvement of TPP in the development of PEM lesions, evaluation of thiamine dependent enzymes and energy substrates in the brain tissues of high dietary S exposed or S-induced PEM cattle should be the focus of future studies.

4) Future study should evaluate the involvement of S and mineral interactions in the development of malacic lesions.

5) Establishment of new maximum tolerable S level for cattle fed DDGS based diets is needed. Direct comparison of S toxicity presenting in DDGS with other high S containing feedstock or high S containing water is needed.

7.3. General Conclusion

The following conclusions can be drawn from the results of the research presented in this thesis:

1) Serum sulfate levels in feedlot steers fed DDGS based diets reflect dietary S intake.
2) Lower serum sulfate levels in finishing steers than backgrounding steers despite high S intake observed in small pen study does not seem to be related to the grain level of the diet.
3) No interaction between dietary S and F:C was observed for S and thiamine metabolism, SCFA absorption or mineral status in beef heifers.
4) Sulfur metabolism and S toxicity in beef heifers were not influenced by F:C.
5) High dietary S reduced DMI but increased ruminal pH.
6) Absorption of SCFA was inhibited by dietary S but increased by low F:C diet.
7) High dietary S did not influence ruminal and blood thiamine and its phosphate esters.
8) Cattle fed high S diet had increased brain TPP and total thiamine while S-induced PEM affected cattle exhibited reduced brain TPP despite abundant total thiamine, suggesting that dietary S may increase the metabolic demand for TPP and that failing to supply enough TPP may lead to the development of PEM lesions in animals affected by S-induced PEM.

9) Low F:C diet increased ruminal thiamine and its phosphate esters but did not influence blood and brain thiamine status.

10) Ruminal and serum Cu, Mo, Zn levels were not influenced by high dietary S.

11) PEM affected brains had reduced Cu, Mo, or Fe concentrations when compared to normal brains, suggesting the possibility of the involvement of the interactions between S and these minerals in the development of PEM lesions.

8.0 REFERENCES

Allen, J. D., and J. M. Gawthorne. 1987. Involvement of the solid phase of rumen digesta in the interaction between copper, molybdenum and sulfur in sheep. Br. J. Nutr. 58(2): 265-276.

Aluwong, T., P. I. Kobo, and A. Abdullahi. 2010. Volatile fatty acids production in ruminants and the role of monocarboxylate transporters: A review. Afr. J. Biotechnol. 9(38): 6229-6232.

Alves de Oliveira, L., C. Jean-Blain, V. Dal Corso, V. Benard, A. Durix, and S. Komisarczuk-Bony. 1996. Effect of a high sulfur diet on rumen microbial activity and rumen thiamine status in sheep receiving a semi-synthetic, thiamine-free diet. Reprod. Nutr. Dev. 36(1): 31-42.

Alves de Oliveira, L., C. Jean-Blain, S. Komisarczuk-Bony, A. Durix, and C. Durier. 1997. Microbial thiamin metabolism in the rumen simulating fermenter (RUSITEC): The effect of acidogenic conditions, a high sulfur level and added thiamin. Br. J. Nutr. 78(4): 599-613.

Amat, S., S. Hendrick, T. A. McAllister, H. C. Block, and J. J. McKinnon. 2012. Effects of distillers' dried grains with solubles from corn, wheat or a 50:50 corn:Wheat blend on performance, carcass characteristics and serum sulfate levels of feedlot steers 3: 343-351.

Ammann, H. M. 1986. A new look at physiologic respiratory response to H2S poisoning. J. Hazard. Mater. 13(3): 369-374.

Anderson, C. M. 1956. The metabolism of sulfur in the rumen of the sheep. N. Z. J. Sci. Technol. 37: 379-394.

Babidge, W., S. Millard, and W. Roediger. 1998. Sulfides impair short chain fatty acid beta-oxidation at acyl-CoA dehydrogenase level in colonocytes: Implications for ulcerative colitis. Mol. Cell. Biochem. 181(1-2): 117-124.

Batal, A., and N. Dale. 2003. Mineral composition of distillers dried grains with solubles. J. Appl. Poult. Res. 12(4): 400-403.

Beke, G. J., and R. Harionaka. 1991. Toxicity to beef cattle of sulfur in saline well water: A case study. Sci. Total. Environ. 101: 281-290.

Belitz, H., W. Grosch, and P. Sheiberle. 2009. 6.Vitaminsin Food Chemistry. 4th ed. Springer-Verlag Berlin Heiderberg.

Beliveau, R. M., and J. J. McKinnon. 2008. Effect of graded levels of wheat-based dried distillers' grains with solubles on performance and carcass characteristics of feedlot steers. Can. J. Anim. Sci. 88(4): 677-684.

Bender, D. A. 2003. Nutritional biochemistry of the vitamins. 2nd Edition. Cambridge University Press. 148-171.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70(2): 567-590.

Bettendorff, L., B. Hennuy, A. De Clerck, and P. Wins. 1994. Chloride permeability of rat brain membrane vesicles correlates with thiamine triphosphate content. Brain Res. 652(1): 157-160.

Bettendorff, L., F. Mastrogiacomo, S. J. Kish, and T. Grisar. 1996. Thiamine, thiamine phosphates, and their metabolizing enzymes in human brain. J. Neurochem. 66(1): 250-258.

Bird, P. R., and I. D. Hume. 1971. Sulfur metabolism and excretion studies in ruminants. IV. cystine and sulfate effects upon the flow of sulfur from the rumen and upon sulfur excretionby sheep.22: 443-452.

Bird, P. R. 1972. Sulfur metabolism and excretion studies in ruminants. X. sulfide toxicity in sheep. Aust. J. Biol. Sci. 25(5): 1087-1098.

Blachier, F., C. Boutry, C. Bos, and D. Tome. 2009. Metabolism and functions of L-glutamate in the epithelial cells of the small and large intestines. Am. J. Clin. Nutr. 90(3): 814S-821S.

Boila, R. J., and L. S. Golfman. 1991. Effects of molybdenum and sulfur on digestion by steers. J. Anim. Sci. 69(4): 1626-1635.

Braunlich, K., and H. Zintzen. 1976. Vitamin B_1 in animal nutrition.Basel: Hoffmann-La Roche. Number: 1593.

Bray, A. C. 1969. Sulfur metabolism in sheep II. the absorption of inorganic sulfate absorption of inorganic sulfate and inorganic sulfide from the sheep’s rumen Aust. J. Agric. Res. 20: 739-749.

Bremer, J., and H. Osmundsen. 1984. Chapter 5 fatty acid oxidation and its regulation. New Compr. Biochem. 7(C): 113-154.

Brent, B. E. 1976. Relationship of acidosis to other feedlot ailments. J. Anim. Sci. 43(4): 930-935.

Brent, B. E., and E. E. Bartley. 1984. Thiamin and niacin in the rumen. J. Anim. Sci. 59(3): 813-822.

Breves, G., H. Hoeller, J. Harmeyer, and H. Martens. 1980. Thiamin balance in the gastrointestinal tract of sheep. J. Anim. Sci. 51(5): 1177-1181.

Buckner, C. D., T. L. Mader, G. E. Erickson, S. L. Colgen, K. K. Karges, and M. L. Gibson. 2007. Optimum concentrations of dry distillers grains with solubles for finishing beef steers. Nebraska Beef Report. MP90: 36-38.

Buckner, C.D., Vanness, S. J., Erickson, G. E., Klopfenstein, T.J.and Benton, J. R. 2008. Sampling wet distillers grains plus solubles to determine NutrientVariability.Nebraska Beef Report: 126-127.

Bulgin, M. S., S. D. Lincoln, and G. Mather. 1996. Elemental sulfur toxicosis in a flock of sheep. J. Am. Vet. Med. Assoc. 208(7): 1063-1065.

Butterworth, R. F. 2003. Thiamin deficiency and brain disorders. Nut. Res. Rev. 16(2): 277-283.

Butterworth, R. F., and A. -. Besnard. 1990. Thiamine-dependent enzyme changes in temporal cortex of patients with alzheimer's disease. Metab. Brain Dis. 5(4): 179-184.

Butterworth, R. F., and M. Heroux. 1989. Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. J. Neurochem. 52(4): 1079-1084.

Cammack, K. M., C. L. Wright, K. J. Austin, P. S. Johnson, R. R. Cockrum, K. L. Kessler et al. 2010. Effects of high-sulfur water and clinoptilolite on health and growth performance of steers fed forage-based diets. J. Anim. Sci. 88(5): 1777-1785.

Care, A. D., R. C. Brown, A. R. Farrar, and D. W. Pickard. 1984. Magnesium absorption from the digestive tract of sheep. Q. J. Exp. Physiol. 69(3): 577-587.

Casley, C. S., L. Canevari, J. M. Land, J. B. Clark, and M. A. Sharpe. 2002. β-Amyloid inhibits integrated mitochondrial respiration and key enzyme activities. J. Neurochem. 80(1): 91-100.

Cebra, C. K., and M. L. Cebra. 2004. Altered mentation caused by polioencephalomalacia, hypernatremia, and lead poisoning. Vet. Clin. North Am. Food Anim. Pract. 20(2): 287-302, vi-vii.

Chiarani, F., C. S. Bavaresco, C. S. Dutra-Filho, C. A. Netto, and A. T. S. Wyse. 2008. Sulfite increases lipoperoxidation and decreases the activity of catalase in brain of rats. Metab. Brain Dis. 23(1): 123-132.

Conrad, H. R., and J. W. Hibbs. 1954. A high roughage system for raising calves based on early rumen development. IV. synthesis of thiamine and riboflavin in the rumen as influenced by the ratio of hay to grain fed and initiation of dry feed consumption. Journal of Dairy Science. 37(5): 512-522.

Cooper, J. R., and J. H. Pincus. 1979. The role of thiamine in nervous tissue. Neurochem. Res. 4(2): 223-239.

Cozannet, P., M. Lessire, J. -. Métayer, C. Gady, Y. Primot, P. -. Geraert et al. 2010. Nutritive value of wheat and maize distillers dried grains with solubles in poultry. Prod. Anim. 23(5): 405-414.

Cramer, S. P., R. Wahl, and K. V. Rajagopalan. 1981. Molybdenum sites of sulfite .. a comparison by EXAFS. J. Am. Chem. Soc. 103(26): 7721-7727.

Cramer, S. P., J. L. Johnson, K. V. Rajagopalan, and T. N. Sorrell. 1979. Observation of 17O effects on MoV EPR spectra in sulfite oxidase; xanthine dehydrogenase, and MoO(SC6H5)4-. Biochem. Biophys. Res. Commun. 91(2): 434-439.

Crawford,G.2007. Managing sulfur concentrations in feed and water." presented at 68th minnesota nutrition conference, minneapolis, september 18-19 (available at http://www.extension.umn.edu/beef/components/pdfs/ManagingSulfur_Crawford.pdf).

Cummings, B. A., D. R. Caldwell, D. H. Gould, and D. W. Hamar. 1995a. Identity and interactions of rumen microbes associated with dietary sulfate-induced polioencephalomalacia in cattle. Am. J. Vet. Res. 56(10): 1384-1389.

Cummings, B. A., D. H. Gould, D. R. Caldwell, and D. W. Hamar. 1995b. Ruminal microbial alterations associated with sulfide generation in steers with dietary sulfate-induced polioencephalomalacia. Am. J. Vet. Res. 56(10): 1390-1395.

Curtis, C. G., T. C. Bartholomew, F. A. Rose, and K. S. Dodgson. 1972. Detoxication of sodium 35S-sulfide in the rat. Biochem. Pharmacol. 21(17): 2313-2316,IN1-IN2,2317-2321.

Davies, E. T., A. H. Pill, D. F. Collings, J. A. Venn, and G. D. Bridges. 1965. Cerebrocortical necrosis in calves. Veterinary Record. 77: 290.

De Sant'Ana, F. J. F., and C. S. L. Barros. 2010. Polioencephalomalacia in ruminants in brazil. Braz. J. Vet. Pathology. 3(1): 70-79.

Dick, A. T. 1956. Molybdenum and copper relationships in animal nutrition. in: McElroy, W.D., glass, B. (eds.), inorganic nitrogen metabolism. john hopkins press, baltimore, MD, USA, pp. 445–473.

Dijkstra, J., H. Boer, J. van Bruchem, M. Bruining, and S. Tamminga. 1993. Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH, and rumen liquid volume. Br. J. Nutr. 69:385–396.

Dougherty, R. W., and H. M. Cook. 1962. Routes of eructated gas expulsion in cattle--a quantitative study. Am. J. Vet. Res. 23: 997-1000.

Doyle, P. T., & R. J. Moir. 1979a. Sulfur and methionine metabolism in sheep. 111. excretion and retention of dietary and supplemented sulfur and production responses to intraruminal infusions of DL-methionine.30: 1185-1196. (Abstr.)

Doyle, P. T., and R. J. Moir. 1979b. Sulfur and methionine metabolism in sheep. I. first approximations of sulfur pools in and sulfur flows from the reticulo-rumen. Aust. J. Biol. Sci. 32(1): 51-63.

Durand, M., and R. Kawashima. 1980. Influence of minerals in rumen microbial digestion. pp. 375-408 in digestive physiol-ogy and metabolism in ruminants. ruckebush and thivend ed., MTP press, ltd., Lancaster, England.

Edwin, E. E., and R. Jackman. 1970. Thiaminase I in the development of cerebrocortical necrosis in sheep and cattle. Nature. 228(5273): 772-774.

Emery, R. S., C. K. Smith, and C. F. Huffman. 1957. Utilization of inorganic sulfate by rumen microorganisms. I. incorporation of inorganic sulfate into amino acids. Appl. Microbiol. 5(6): 360-362.

Ensminger, M. E., J. E. Oldfield, and W. W. Heinemann eds. 1990. Feeds and Nutrition. 2nd ed. Ensminger Publishing Co. Clovis, California.

Farrer, K. T. 1947. The thermal destruction of vitaminB. Biochem. J. 41: 167-169.

Felix, T. L., and S. C. Loerch. 2011. Effects of haylage and monensin supplementation on performance, carcass characteristics, and ruminal metabolism of feedlot cattle fed diets containing 60% dried distillers grains. J. Anim. Sci. 89(8): 2614-2623.

Ferreira, H. G., F. A. Harrison, and R. D. Keynes. 1966a. The potential and short-circuit current across isolated rumen epithelium of the sheep. The Journal of Physiology. 187(3): 631-644.

Ferreira, H. G., F. A. Harrison, R. D. Keynes, and A. H. Nauss. 1966b. Observations on the potential across the rumen of the sheep. The Journal of Physiology. 187(3): 615-630.

Firkins, J. L. 1996. Maximizing microbial protein synthesis in the rumen. J. Nutr. 126(4 Suppl): 1347S-54S.

Hicks, R. B., F. N. Owens, D. R. Gill, J. J. Martin, and C. A. Strasia. 1988. Water intake by feedlot steers, 208. Oklahoma Animal Science Report Mp-125. Stillwater, Okla.: Oklahoma State University

Gäbel, G., F. Müller, H. Pfannkuche, and J. R. Aschenbach. 2001. Influence of isoform and DNP on butyrate transport across the sheep ruminal epithelium. J. Comp. Physiol. B 171:215-221.

Gawthorne, J. M., and C. J. Nader. 1976. The effect of molybdenum on the conversion of sulfate to sulfide and microbial protein sulfur in the rumen of sheep. Br. J. Nutr. 35(1): 11-23.

Gibson, G. E., K. -. R. Sheu, J. P. Blass, A. Baker, K. C. Carlson, B. Harding et al. 1988. Reduced activities of thiamine-dependent enzymes in the brains and peripheral tissues of patients with alzheimer's disease. Arch. Neurol. 45(8): 836-840.

Gilmour, B. 1986. Potential market impact of a Corn/Ethanol plant to be located in Southern Ontario, Agriculture Canada.

Goetsch, A. L., and F. N. Owens. 1987. Effects of supplement sulfate (dynamate) and thiamin-HCl on passage of thiamin to the duodenum and site of digestion in steers. Arch. Tierernahr. 37(12): 1075-1083.

Gooneratne, S. R., B. Laarveld, K. K. Pathirana, and D. A. Christensen. 2011. Effects of dietary cu, mo and S on urinary cu and zn excretion in simmental and angus cattle. Res. Vet. Sci. 91(3): 116-20.

Gooneratne, S. R., A. A. Olkowski, and D. A. Christensen. 1989a. Sulfur-induced polioencephalomalacia in sheep: Some biochemical changes. Can. J. Vet. Res. 53(4): 462-467.

Gooneratne, S. R., A. A. Olkowski, R. G. Klemmer, G. A. Kessler, and D. A. Christensen. 1989b. High sulfur related thiamine deficiency in cattle: A field study. Can. Vet. J. 30(2): 139-146.

Gould, D. H. 2000. Update on sulfur-related polioencephalomalacia. Vet. Clin. North Am. Food Anim. Pract. 16(3): 481-96, vi-vii.

Gould, D. H. 1998. Polioencephalomalacia. J. Anim. Sci. 76(1): 309-314.

Gould, D. H., B. A. Cummings, and D. W. Hamar. 1997. In vivo indicators of pathologic ruminal sulfide production in steers with diet-induced polioencephalomalacia. J. Vet. Diagn. Invest. 9(1): 72-76.

Gould, D. H., M. M. McAllister, J. C. Savage, and D. W. Hamar. 1991. High sulfide concentrations in rumen fluid associated with nutritionally induced polioencephalomalacia in calves. Am. J. Vet. Res. 52(7): 1164-1169.

Ham, G. A., R. A. Stock, T. J. Klopfenstein, E. M. Larson, D. H. Shain, and R. P. Huffman. 1994. Wet corn distillers byproducts compared with dried corn distillers grains with solubles as a source of protein and energy for ruminants. J. Anim. Sci. 72(12): 3246-3257.

Hansard, S. L., and A. S. Mohammed. 1969. Absorption, excretion, and maternal-fetal utilization of sulfate by the bovine. J. Anim. Sci. 28(2): 283-287.

Harmeyer, J., and U. Kollenkirchen. 1989. Thiamin and niacin in ruminant nutrition. Nutr. Res. Rev. 2(1): 201-225.

Harper, C. 2006. Thiamine (vitamin B1) deficiency and associated brain damage is still common throughout the world and prevention is simple and safe! Eur. J. Neurol. 13(10): 1078-1082.

Harries, N. 1987. Polioencephalomalacia in feedlot cattle drinking water high in sodium sulfate. Can. Vet. J. 28: 717.

Haydock, D. 2003. Sulfur-induced polioencephalomalacia in a herd of rotationally grazed beef cattle. Can. Vet. J. 44(10): 828-829.

Hayes, B. W., G. E. Mitchell, J. C. O. Little, and N. W. Bradley. 1966. Concentrations of B-vitamins in ruminal fluid of steers fed different levels and physical forms of hay and grain. J. Anim. Sci. 25: 539-542.

Heroux, M., V. L. Raghavendra Rao, J. Lavoie, J. S. Richardson, and R. F. Butterworth. 1996. Alterations of thiamine phosphorylation and of thiamine-dependent enzymes in alzheimer's disease. Metab. Brain Dis. 11(1): 81-88.

Hill, C. H. 1969. A role of copper in elastin formation. Nutr. Rev. 27(4): 99-100.

Hoeller, H., M. Fecke, and K. Schaller. 1977. Permeability to thiamin of the sheep rumen wall in vitro. J. Anim. Sci. 44(1): 158-161.

Hooser, S. B., W. Van Alstine, M. Kiupel, and J. Sojka. 2000. Acute pit gas (hydrogen sulfide) poisoning in confinement cattle. J. Vet. Diagn. Invest. 12(3): 272-275.

Huisingh, J., D. C. Milholland, and G. Matrone. 1975. Effect of molybdate on sulfide production from methionine and sulfate by ruminal microorganisms of sheep. J. Nutr. 105(9): 1199-1205.

Hunt, C. H., O. G. Bentley, T. V. Hershberger, and J. H. Cline. 1954. The effect of carbohydrates and sulfur on B-vitamins synthesis, cellulose digestion, and urea utilization by rumen microorganisms in vitro. J. Animal Sci. 13: 570-579.

Ivancic Jr., J., and W. P. Weiss. 2001. Effect of dietary sulfur and selenium concentrations on selenium balance of lactating holstein cows. J. Dairy Sci. 84(1): 225-232.

Iwata, H., Y. Yabushita, T. Doi, and T. Matsuda. 1985. Synthesis of thiamine triphosphate in rat brain in vivo. Neurochem. Res. 10(6): 779-787.

Jeffrey, M., J. P. Duff, R. J. Higgins, V. R. Simpson, R. Jackman, T. O. Jones et al. 1994. Polioencephalomalacia associated with the ingestion of ammonium sulfate by sheep and cattle. Vet. Rec. 134(14): 343-348.

Jensen, R., O. R. Adams, and L. A. Griner. 1956. Polioencephalomalacia of cattle and sheep. J. Am. Vet. Med. Assoc. 129(7): 311-321.

Johnson, C. L., and D. A. Aubrey Jones. 1989. Effect of change of diet on the mineral composition of rumen fluid, on magnesium metabolism and on water balance in sheep. Br. J. Nutr. 61(3): 583-594.

Johnson, S. 2001. The multifaceted and widespread pathology of magnesium deficiency. Med. Hypotheses. 56(2): 163-170.

Jørgensen, J., and P. B. Mortensen. 2001. Hydrogen sulfide and colonic epithelial metabolism: Implications for ulcerative colitis. Dig. Dis. Sci. 46(8): 1722-1732.

Kasahara, T., S. Ichijo, S. Osame, and T. Sarashina. 1989. Clinical and biochemical findings in bovine cerebrocortical necrosis produced by oral administration of amprolium. Nihon Juigaku Zasshi. 51(1): 79-85.

Kennedy, P. M., E. R. Williams, and B. D. Siebert. 1975. Sulfate recycling and metabolism in sheep and cattle. Aust. J. Biol. Sci. 28(1): 31-42.

Khan, S. U., G. E. Morris, and M. Hidiroglou. 1980. Rapid estimation of sulfide in rumen and blood with a sulfide specific ion electrode. Microchem. J. 25: 388-395.

Kish, S. J., F. Mastrogiacomo, M. Guttman, Y. Furukawa, J. -. Taanman, S. Dožić et al. 1999. Decreased brain protein levels of cytochrome oxidase subunits in alzheimer's disease and in hereditary spinocerebellar ataxia disorders: A nonspecific change J. Neurochem. 72(2): 700-707.

Kitajima, S., S. Takuma, and M. Morimoto. 1999. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. Exp. Anim. 48(3): 137-143.

Knight, C. W., K. C. Olson, C. L. Wright, K. J. Austin, K. M. Cammack, and R. Cockrum. 2008. Sulfur-induced polioencephalomalacia in roughage-fed feedlot steers administered high-sulfur water. West. Sec. Amer. Soc. Anim. Sci. 59: 364-366.

Koenig, K. M., L. M. Rode, R. D. H. Cohen, and W. T. Buckley. 1997. Effects of diet and chemical form of selenium on selenium metabolism in sheep. J. Anim. Sci. 75(3): 817-827.

Kul, O., S. Karahan, M. Basalan, and N. Kabakci. 2006. Polioencephalomalacia in cattle: A consequence of prolonged feeding barley malt sprouts. J. Vet. Med. A Physiol. Pathol. Clin. Med. 53(3): 123-128.

Kung, J. L., J. P. Bracht, A. O. Hession, and J. Y. Tavares. 1998. High sulfate inducedpolioencephalomalacia (PEM) in cattle – burping can be dangerous if you are a ruminant.Pacific Northwest Nutrition Conference, Vancouver, BC, Canada.

Laforenza, U., C. Patrini, and G. Rindi. 1988. Distribution of thiamine, thiamine phosphates, and thiamine metabolizing enzymes in neuronal and glial cell enriched fractions of rat brain. J. Neurochem. 51(3): 730-735.

Lardinois, C. C., R. C. Mills, C. A. Elvehjem, and A. B. Hart. 1944. Rumen synthesis of the vitamin B complex as influenced by ration composition. Journal of Dairy Science. 27(7): 579-583.

Leichter, J., and M. A. Joslyn. 1969. Kinetics of thiamin cleavage by sulfite. Biochem. J. 113(4): 611-615.

Lenz, A.and H. Holzer. 1985. Cleavage of thiamine pyrophosphate by sulfite in saccharomyces cerevisiae. Z Lebensm Unters Forch. 181(5): 417-421.

Leschelle, X., M. Goubern, M. Andriamihaja, H. M. Blottiere, E. Couplan, M. D. Gonzalez-Barroso et al. 2005. Adaptative metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide. Biochim. Biophys. Acta. 1725(2): 201-212.

Leupp, J. L., G. P. Lardy, M. L. Bauer, K. K. Karges, M. L. Gibson, J. S. Caton et al. 2009. Effects of distillers dried grains with solubles on growing and finishing steer intake, performance, carcass characteristics, and steak color and sensory attributes. J. Anim. Sci. 87(12): 4118-4124.

Lewis, D. 1954. The reduction of sulfate in the rumen of the sheep. Biochem. J. 56(3): 391-399.

Lindqvist, Y., G. Schneider, U. Ermler, and M. Sundstrom. 1992. Three-dimensional structure of transketolase, a thiamine diphosphate dependent enzyme, at 2.5Å resolution. EMBO J. 11(7): 2373-2379.

Liu, J. -., and T. D. Hurley. 2011. A new crystal form of mouse thiamin pyrophosphokinase. Intl. J. Biochem. Mol. Biol. 2(2): 111-118.

Lodemann, U., and H. Martens. 2006. Effects of diet and osmotic pressure on na+ transport and tissue conductance of sheep isolated rumen epithelium. Exp. Physiol. 91(3): 539-550.

Loew, F. M., and R. H. Dunlop. 1972. Induction of thiamine inadequacy and polioencephalomalacia in adult sheep with amprolium. Am. J. Vet. Res. 33(11): 2195-2205.

Loneragan, G. H., D. H. Gould, R. J. Callan, C. J. Sigurdson, and D. W. Hamar. 1998. Association of excess sulfur intake and an increase in hydrogen sulfide concentrations in the ruminal gas cap of recently weaned beef calves with polioencephalomalacia. J. Am. Vet. Med. Assoc. 213(11): 1599-604, 1571.

Loneragan, G. H., J. J. Wagner, D. H. Gould, F. B. Garry, and M. A. Thoren. 2001. Effects of water sulfate concentration on performance, water intake, and carcass characteristics of feedlot steers. J. Anim. Sci. 79(12): 2941-2948.

Lonsdale, D. 2006. A review of the biochemistry, metabolism and clinical benefits of thiamin(e) and its derivatives. Evid. -Based Complement. Altern. Med. 3(1): 49-59.

Martens, H., and I. Blume. 1986. Effect of intraruminal sodium and potassium concentrations and of the transmural potential difference on magnesium absorption from the temporarily isolated rumen of sheep. Q. J. Exp. Physiol. 71(3): 409-415.

Mastrogiacomo, F., L. Bettendorff, T. Grisar, and S. J. Kish. 1996a. Brain thiamine, its phosphate esters, and its metabolizing enzymes in Alzheimer's disease. Ann. Neurol. 39(5): 585-591.

Mastrogiacomo, F., J. G. Lindsay, L. Bettendorff, J. Rice, and S. J. Kish. 1996b. Brain protein and α-ketoglutarate dehydrogenase complex activity in alzheimer's disease. Ann. Neurol. 39(5): 592-598.

McDowell, L. R. 2000. Vitamins in animal and human nutrition. 2nd Edition ed. Ames, IA, Iowa State University Press.

McDowell, L. R. 1992. Minerals in animal and human nutrition. academic press inc. harcourt brace jovanovich publishers, san diego, CA.

Mikus, J. H. 2004. Effect of inorganic and organically complexed trace minerals on growing cattle consuming drinking water with high levels of sulfate. Doctoral dissertation, Texas Tech University.

Milad, K. E., and G. S. Ridha. 2009. The occurrence of thiamine-responsive polioencephalomalacia in dromedary breeding camels in Libya: Preliminary investigation of diagnosis. Iraqi Journal of Veterinary Sciences. 23(Supplement I): 119-122.

Miller, B. L., R. D. Goodrich, and J. C. Meiske. 1986. Effects of monensin and concentrate percentage on B-vitamin production in steers. J. Anim. Sci. 62: 484-496.

Mills, C. F. 1960. Comparative studies of copper, molybdenum and sulfur metabolism in the ruminant and the rat. Proc. Nutr. Soc. 19: 162-169.

Mizuno, Y., S. -. Ikebe, N. Hattori, H. Mochizuki, Y. Nakagawa-Hattori, and T. Kondo. 1994a. Studies on the pathogenesis of Parkinson's disease in Japan. Arch. Gerontol. Geriatr. 19(2): 105-121.

Mizuno, Y., T. Kondo, and H. Mori. 1994b. Various aspects of motor fluctuations and their management in parkinson's disease. Neurology. 44(7 Suppl 6): S29-34.

Moore, J. W. E., S. Millard, W. Babidge, R. Rowland, and W. E. W. Roediger. 1997. Hydrogen sulfide produces diminished fatty acid oxidation in the rat colon in vivo: Implications for ulcerative colitis. AUST. NEW ZEALAND J. SURG. 67(5): 245-249.

Murakami, S., S. Kurihara, N. Koikawa, A. Nakamura, K. Aoki, H. Yosigi et al. 2009. Effects of oral supplementation with cystine and theanine on the immune function of athletes in endurance exercise: Randomized, double-blind, placebo-controlled trial. Biosci. Biotechnol. Biochem. 73(4): 817-821.

Murata, K. 1982. Actions of two types of thiaminase on thiamin and its analogues. Ann. N. Y. Acad. Sci. 378: 146-156.

Mustafa, A. F., J. J. McKinnon, and D. A. Christensen. 2000. The nutritive value of thin stillage and wet distillers' grains for ruminants: Review. Asian-Australas. J. Anim. Sci. 13(11): 1609-1618.

Neville, B. W., G. P. Lardy, K. K. Karges, L. A. Kirschten, and C. S. Shauer. 2011. Sulfur balance in lambs fed increasing concentrations of distillers dried grains with solubles. SheepResearch Report. : 23-25.

Neville, B. W., G. P. Lardy, K. K. Karges, S. R. Eckerman, P. T. Berg, and C. S. Schauer. 2012. Interaction of corn processing and distillers dried grains with solubles on health and performance of steers. J. Anim. Sci. 90(2): 560-567.

Neville, B. W., C. S. Schauer, K. Karges, M. L. Gibson, M. M. Thompson, L. A. Kirschten et al. 2010. Effect of thiamine concentration on animal health, feedlot performance, carcass

characteristics, and ruminal hydrogen sulfide concentrations in lambs fed diets based on 60% distillers dried grains plus solubles. J. Anim. Sci. 88(7): 2444-2455.

Niles, G. A., S. E. Morgan, and W. C. Edwards. 2002a. The relationship betweensulfur, thiamine and polioencephalomalacia — a review.Bov Pract.36: 93-99.

Niles, G. A., S. Morgan, W. C. Edwards, and D. Lalman. 2002b. Effects of dietary sulfur concentrations on the incidence and pathology of polioencephalomalicia in weaned beef calves. Vet. Hum. Toxicol. 44(2): 70-72.

Niles, G. A., S. E. Morgan, and W. C. Edwards. 2000. Sulfur-induced polioencephalomalacia in stocker calves. Vet. Hum. Toxicol. 42(5): 290-291.

Nordlund, K. W. 2003. Herd-based diagnosis of subacute ruminal acidosis. American Association of Bovine Practitioners 36th Annual Conference Seminar 7:Dairy Herd Problem Investigation Strategies.

NRC. 2005. Requirements of beef cattle. Washington, D.C, National Academy Press.

NRC. 2000. Nutrient requirements of beef cattle. Washington,D.C, National Academy Press.

NRC. 1996. Nutrient requirements of beef cattle. Washington, D.C., National Academy Press.

NRC. 2007. Nutrient requirements of small ruminants. 6th rev. ed. Washington,D.C, National Academy Press.

O'Brien, J., and R. D. Vetter. 1990. Production of thiosulfate during sulfide oxidation by mitochondria of the symbiont-containing bivalve solemya reidi. J. Exp. Biol. 149: 133-148.

Olkowski, A. A. 2009. Livesotck water quality: A field guide for cattle, horses, poultry and swine. . Ottawa, Her Majestythe Queen in Right of Canada.

Olkowski, A. A., C. G. Rousseaux, and D. A. Christensen. 1991. Association of sulfate-water and blood thiamine concentration in beef cattle: Field studies. Can. J. Anim. Sc. 71: 825-832.

Olkowski, A. A. 1992. Nutritional toxicity of sulfur and sulfur-nutrient interactions in ruminants. Doctoral dissertation, University of Saskatchewan.

Olkowski, A. A. 1997. Neurotoxicity and secondary metabolic problems associated with low to moderate levels of exposure to excess dietary sulfur in ruminants: A review. Vet. Hum. Toxicol. 39(6): 355-360.

Olkowski, A. A., and H. L. Classen. 1996. The study of thiamine requirement in broiler chickens. Int. J. Vitam. Nutr. Res. 66(4): 332-341.

Olkowski, A. A., S. R. Gooneratne, C. G. Rousseaux, and D. A. Christensen. 1992. Role of thiamine status in sulfur induced polioencephalomalacia in sheep. Res. Vet. Sci. 52(1): 78-85.

Olkowski, A. A., B. Laarveld, J. F. Patience, S. I. Francis, and D. A. Christensen. 1993a. The effect of sulfate on thiamine-destroying activity in rumen content cultures in-vitro. Int. J. Vitam. Nutr. Res. 63(1): 38-44.

Olkowski, A. A., B. Laarveld, J. F. Patience, S. I. Francis, and D. A. Christensen. 1993b. The effect of sulfate on thiamine-destroying activity in rumen content cultures in-vitro. Int. J. Vitam. Nutr. Res. 63(1): 38-44.

Palus, V., J. Penderis, S. Jakovljevic, and G. B. Cherubini. 2010. Thiamine deficiency in a cat: Resolution of MRI abnormalities following thiamine supplementation. J. Feline Med. Surg. 12(10): 807-810.

Pannunzio, P., A. S. Hazell, M. Pannunzio, K. V. Rama Rao, and R. F. Butterworth. 2000. Thiamine deficiency results in metabolic acidosis and energy failure in cerebellar granule cells: An in vitro model for the study of cell death mechanisms in wernicke's encephalopathy. J. Neurosci. Res. 62(2): 286-292.

Penner, G. B., and J. R. Aschenbech. 2011. Mechanisms of acid absorption in the rumen and impacts on subacute rumen acidosis. Proceedings of the 22nd Annual Florida Ruminant Nutrition Symposium. : 90-104.

Penner, G. B., K. A. Beauchemin, and T. Mutsvangwa. 2007. Severity of ruminai acidosis in primiparous holstein cows during the periparturient period. J. Dairy Sci. 90(1): 365-375.

Penner, G. B., K. A. Beauchemin, and T. Mutsvangwa. 2006. An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. J. Dairy Sci. 89(6): 2132-2140.

Penner, G. B., M. A. Steele, J. R. Aschenbach, and B. W. McBride. 2011. Ruminant nutrition symposium: Molecular adaptation of ruminal epithelia to highly fermentable diets. J. Anim. Sci. 89(4): 1108-1119.

Penner, G. B., M. Taniguchi, L. L. Guan, K. A. Beauchemin, and M. Oba. 2009. Effect of dietary forage to concentrate ratio on volatile fatty acid absorption and the expression of genes related to volatile fatty acid absorption and metabolism in ruminal tissue. J. Dairy Sci. 92(6): 2767-2781.

Pitcher, M. C. L., and J. H. Cummings. 1996. Hydrogen sulfide: A bacterial toxin in ulcerative colitis? Gut. 39(1): 1-4.

Pope, A. L., R. J. Moir, M. Somers, E. J. Underwood, and C. L. White. 1979. The effect of sulfur on se absorption and retention in sheep.109: 1448-1455.

Powell, M. A., and G. N. Somero. 1986. Hydrogen sulfide oxidation is coupled to oxidative phosphorylation in mitochondria of solemya reidi. Science. 233(4763): 563-566.

Qi, K., C. D. Lu, and F. N. Owens. 1993. Sulfate supplementation of angora goats: Sulfur metabolism and interactions with zinc, copper and molybdenum. Small Ruminant Res. 11(3): 209-225.

Rachid, M. A., E. F. Filho, A. U. Carvalho, A. C. Vasconcelos, and P. M. Ferreira. 2011. Polioencephalomalacia in cattle. Asian Journal of Animal and Veterinary Advances. 6(2): 126-131.

Raghavendra Rao, V. L., J. S. Richardson, and R. F. Butterworth. 1993. Decreased activities of thiamine diphosphatase in frontal and temporal cortex in Alzheimer's disease. Brain Res. 631(2): 334-336.

Raisbeck, M. F. 1982. Is polioencephalomalacia associated with high-sulfate diets? J. Am. Vet. Med. Assoc. 180(11): 1303-1305.

Ramana, G. V., P. Veera Bramhachari, and K. V. Chaitanya. 2012. Modeling of arabidopsis and soybean thiamine pyrophospho kinase genes. Journal of Advanced Bioinformatics Applications and Research. 3 (2): 294-303.

Rammell, C. G., and J. H. Hill. 1986. A review of thiamine deficiency and its diagnosis, especially in ruminants. N. Z. Vet. J. 34(12): 202-204.

Ramos, J. J., L. M. Ferrer, L. Garcia, A. Fernandez, and A. Loste. 2005. Polioencephalomalacia in adult sheep grazing pastures with prostrate pigweed. Can. Vet. J. 46(1): 59-61.

Ramos, J. J., C. Marca, A. Loste, J. A. Garcia de Jalon, A. Fernandez, and T. Cubel. 2003. Biochemical changes in apparently normal sheep from flocks affected by polioencephalomalacia. Vet. Res. Commun. 27(2): 111-124.

Reist, M., K. -. Marshall, P. Jenner, and B. Halliwell. 1998. Toxic effects of sulfite in combination with peroxynitrite on neuronal cells. J. Neurochem. 71(6): 2431-2438.

Requejo, R., T. R. Hurd, N. J. Costa, and M. P. Murphy. 2010. Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage. FEBS J. 277(6): 1465-1480.

Richter, E. L., M. E. Drewnoski, and S. L. Hansen. 2012. Effects of increased dietary sulfur on beef steer mineral status, performance, and meat fatty acid composition. J. Anim. Sci. 90(11):3945-53

Rindi, G., and U. Laforenza. 2000. Thiamine intestinal transport and related issues: Recent aspects. Proc. Soc. Exp. Biol. Med. 224(4): 246-255.

Roberts, G. W., and J. W. Boyd. 1974. Cerebrocortical necrosis in ruminants. occurrence of thiaminase in the gut of normal and affected animals and its effect on thiamine status. J. Comp. Pathol. 84(3): 365-374.

Roediger, W. E., A. Duncan, O. Kapaniris, and S. Millard. 1993a. Reducing sulfur compounds of the colon impair colonocyte nutrition: Implications for ulcerative colitis. Gastroenterology. 104(3): 802-809.

Roediger, W. E., A. Duncan, O. Kapaniris, and S. Millard. 1993b. Sulfide impairment of substrate oxidation in rat colonocytes: A biochemical basis for ulcerative colitis? Clin. Sci. (Lond). 85(5): 623-627.

Roediger, W. E., J. Moore, and W. Babidge. 1997. Colonic sulfide in pathogenesis and treatment of ulcerative colitis. Dig. Dis. Sci. 42(8): 1571-1579.

Rousseaux, C. G., A. A. Olkowski, A. Chauvet, S. R. Gooneratne, and D. A. Christenson. 1991. Ovine polioencephalomalacia associated with dietary sulfur intake. Zentralbl. Veterinarmed. A. 38(3): 229-239.

Russo, V. M., and S. V. Karmarker. 1998. Water extraction of plant tissues for analysis by ion chromatography. Commun. Soil Sci. Plant Anal. 29: 245253. 29: 245-253.

Said, H. M., A. Ortiz, G. K. Kumar, N. Chatterjee, P. K. Dudeja, and S. Rubin. 1999. Transport of thiamine in human intestine: Mechanism and regulation in intestinal epithelial cell model caco-2. Am. J. Physiol. Cell Physiol. 277(4 46-4): C645-C651.

Sakhaee, E., and A. Derakhshanfar. 2010. Polioencephalomalacia associated with closantel overdosage in a goat. J. S. Afr. Vet. Assoc. 81(2): 116-117.

Sarturi, J. O., G. E. Erickson, T. J. Klopfenstein, J. T. Vasconcelos, K. Rolfe, and M. G. Dib. 2011. Effects of sulfur content of wet or dry distillers grains in beef cattle finishing diets on intake, ruminal pH, and hydrogen sulfide.Nebraska Beef Cattle Report: Page 68.

Schweizer, U., A. U. Bräuer, J. Köhrle, R. Nitsch, and N. E. Savaskan. 2004. Selenium and brain function: A poorly recognized liaison. Brain Res. Rev. 45(3): 164-178.

Shaw, L., and P. C. Engel. 1987. CoA-persulfide: A possible in vivo inhibitor of mammalian short-chain acyl-CoA dehydrogenase. BIOCHIM. BIOPHYS. ACTA LIPIDS LIPID METAB. 919(2): 171-174.

Smart, M. E., C. Cohen, D. A. Christensen, and C. M. Williams. 1986. The effects of sulfate removal from the drinking water on plasma and livercopper and zinc concentrations of beef cows and their calves. Can. J. Anim. 66: 669-680.

Smiricky-Tjardes, M. R., I. Mavromichalis, D. M. Albin, J. E. Wubben, M. Rademacher, and V. M. Gabert. 2004. Bioefficacy of L-lysine sulfate compared with feed-grade L-lysine·HCl in young pigs. J. Anim. Sci. 82(9): 2610-2614.

Smith, D. F., and D. O. Keppler. 1977. 2-deoxy-D-galactose metabolism in ascites hepatoma cells results in phosphate trapping and glycolysis inhibition. Eur. J. Biochem. 73(1): 83-92.

Spears, J. W. 2003. Trace mineral bioavailability in ruminants. J. Nutr. 133(5 Suppl 1): 1506S-9S.

Spears, J. W., J. H. Clark, and E. E. Hatfield. 1985. Nitrogen utilization and ruminal fermentation in steers fed soybean meal treated with formaldehyde. J. Anim. Sci. 60(4): 1072-1080.

Spiehs, M. J., and V. H. Varel. 2009. Nutrient excretion and odorant production in manure from cattle fed corn wet distillers grains with solubles. J. Anim. Sci. 87(9): 2977-2984.

Standish, J. F., and C. B. Ammerman. 1971. Effect of excess dietary iron as ferrous sulfate and ferric citrate on tissue mineral composition of sheep. J. Anim. Sci. 33(2): 481-484.

Stevens, C. E., and B. K. Stettler. 1966. Factors affecting the transport of volatile fatty acids across rumen epithelium. Am. J. Physiol. 210(2): 365-372.

Suárez, B. J., C. G. Van Reenen, G. Beldman, J. Van Delen, J. Dijkstra, and W. J. J. Gerrits. 2006. Effects of supplementing concentrates differing in carbohydrate composition in veal calf diets: I. animal performance and rumen fermentation characteristics. J. Dairy Sci. 89(11): 4365-4375.

Suttle, N. F., P. Abrahams, and I. Thornton. 1984. The role of a soil x dietary sulfur interaction in the impairment of copper absorption by ingested soil in sheep. J. Agric. Sci. 103: 81-86.

Suttle, N. F. 2010. Mineral nutrition of livestock. 4th ed.CABI.

Suttle, N. F. 1991. The interactions between copper, molybdenum, and sulfur in ruminant nutrition. Annu. Rev. Nutr. 11: 121-140.

Suttle, N. F. 1975. The role of organic sulfur in the copper-molybdenum-S interrelationship in ruminant nutrition. Br. J. Nutr. 34(3): 411-420.

Suttle, N. F. 1974a. Effects of organic and inorganic sulfur on the availability of dietary copper to sheep. Br. J. Nutr. 32(3): 559-568.

Suttle, N. F. 1974b. Recent studies of the copper-molybdenum antagonism. Proc. Nutr. Soc. 33(3): 299-305.

Suttle, N. F., and A. C. Field. 1983. Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. J. Comp. Pathol. 93(3): 379-389.

Tallaksen, C. M., H. Bell, and T. Bohmer. 1993. Thiamin and thiamin phosphate ester deficiency assessed by high performance liquid chromatography in four clinical cases of wernicke encephalopathy. Alcohol. Clin. Exp. Res. 17(3): 712-716.

Tallaksen, C. M., H. Bell, and T. Bohmer. 1992. The concentration of thiamin and thiamin phosphate esters in patients with alcoholic liver cirrhosis. Alcohol Alcohol. 27(5): 523-530.

Tanphaichair, V. 1976. Thiamine. in "nutrition reviews present knowledge in nutrition" (fourth ed.) (D.M. hegsted, C.O. chichester, W.J. darby, K.W. McNutt, R.M. stalvey, and E.H. stotz, eds.). .New York, The Nutrition Foundation Inc.

Terlecki, S., and L. M. Markson. 1961. Cerebrocortical necrosis in cattle and sheep. Vet. Rec. 73: 23-27.

Thomson, A. D., P. R. Ryle, G. K . Shaw. 1983. Ethanol, thiamine and brain damage. Alcohol Alcohol 18:27-43.

Thornber, E. J., R. H. Dunlop, and J. M. Gawthorne. 1980. Thiamin deficiency in the lamb: Changes in thiamin phosphate esters in the brain. J. Neurochem. 35(3): 713-717.

Thornber, E. J., R. H. Dunlop, J. M. Gawthorne, and C. R. Huxtable. 1981. Induced thiamin deficiency in lambs. Aust. Vet. J. 57(1): 21-26.

Thornber, E. J., R. H. Dunlop, J. M. Gawthorne, and C. R. Huxtable. 1979. Polioencephalomalacia (cerebrocortical necrosis) induced by experimental thiamine deficiency in lambs. Res. Vet. Sci. 26(3): 378-380.

Todd, K., and R. Butterworth. 1999. Mechanisms of selective neuronal cell death due to thiamine deficiency. Ann N Y Acad Sci. 893: 404-411.

Underwood, B. A., and S. Smitasiri. 1999. Micronutrient malnutrition: Policies and programs for control and their implications. Annu. Rev. Nutr. 19: 303-324.

Uwituze, S., G. L. Parsons, K. K. Karges, M. L. Gibson, L. C. Hollis, J. J. Higgins et al. 2011a. Effects of distillers grains with high sulfur concentration on ruminal fermentation and digestibility of finishing diets. J. Anim. Sci. 89(9): 2817-2828.

Uwituze, S., G. L. Parsons, C. J. Schneider, K. K. Karges, M. L. Gibson, L. C. Hollis et al. 2011b. Evaluation of sulfur content of dried distiller's grains with solubles in finishing diets based on steam-flaked corn or dry-rolled corn. J. Anim. Sci. 89(8): 2582-2591.

Van Ryssen, J. B. J., P. S. M. Van Malsen, and F. Hartmann. 1998. Contribution of dietary sulfur to the interaction between selenium and copper in sheep. J. Agric. Sci. 130(1): 107-114.

Vanness, S., N. Meyer, T. J. Klopfenstein, and G. E. Erickson. 2009. Ruminal sulfide levels in corn byproduct diets with varying roughage levels.Nebraska Beef Cattle Reports. Paper 540.

Walter, L. J., J. L. Aalhus, W. M. Robertson, T. A. McAllister, D. J. Gibb, M. E. R. Dugan et al. 2010. Evaluation of wheat or corn dried distillers' grains with solubles on performance and carcass characteristics of feedlot steers. Can. J. Anim. Sci. 90(2): 259-269.

Ward, G. M. 1978. Molybdenum toxicity and hypocuprosis in ruminants: A review. J. Anim. Sci. 46(4): 1078-1085.

Weigel, J. C., D. Loy, and L. Kilmer. 1997. Feed co-products of the dry corn milling process. iowa state university, iowa corn promotion board, iowa department of agriculture, renewable fuels association, national corn growers association.available online at http://www.iowacorn.org/forms/drymillbook.pdf.

Weir, W. C., and V. V. Rendig. 1954. Serum inorganic sulfate sulfur as a measure of the sulfur intake of sheep. J. Nutr. 54(1): 87-96.

Weiss, W. P and N. St-Pierre. 2010. Feeding strategies to decrease manure output of dairy cows. WCDS Advances in Dairy Technology. Volume 22: 229-237

Whanger, P. D., O. H. Muth, J. E. Oldfield, and P. H. Weswig. 1969. Influence of sulfur on incidence of white muscle disease in lambs. J. Nutr. 97(4): 553-562.

White, C. L., and M. Somers. 1977. Sulfur selenium studies in sheep. I. the effects of varying dietary sulfate and selenomethionine on sulfur, nitrogen and selenium metabolism in sheep. Aust. J. Biol. Sci. 30(47): 56.

White, P. J., H. C. Bowen, B. Marshall, and M. R. Broadley. 2007. Extraordinarily high leaf selenium to sulfur ratios define 'Se-accumulator' plants Ann Bot. 100(1): 111-118.

Widmann, M., R. Jurgen, and J. Pleiss. 2010. The thiamine diphosphate dependent enzyme engineering database: A tool for the systematic analysis of sequence and structure relations .BMC Biochem. 11 (1):9

Williams, R. R., R. E. Waterman, J. C. Keresztesy, and E. R. Buchman. 1935. Studies of crystalline vitamin B. III. cleavage of vitamin with sulfite. J. Arh. Chem. Sot. 57: 536-537.

Willis, C. L., J. H. Cummings, G. Neale, and G. R. Gibson. 1997. Nutritional aspects of dissimilatory sulfate reduction in the human large intestine. Curr. Microbiol. 35(5): 294-298.

Wright, C. L. 2007. Management of water quality for beef cattle. Vet. Clin. North Am. Food Anim. Pract. 23(1): 91-103.

Yang, W. Z., Y. L. Li, T. A. McAllister, J. J. McKinnon, and K. A. Beauchemin. 2012. Wheat distillers grains in feedlot cattle diets: Feeding behavior, growth performance, carcass characteristics, and blood metabolites. J. Anim. Sci. 90(4): 1301-1310.

Zhang, Y. 2010. Sulfur concentration in distiller's dried grains with soluble (DDGS) and its impact on palatability and pig performance. animal science--nutritional efficency consortum. national corn-to-ethanol research CenterResearch report. Animal Science--Nutritional Efficency Consortum. National Corn-to-Ethanol Research Center Research Report

Zhang, X., A. S. Vincent, B. Halliwell, and K. P. Wong. 2004. A mechanism of sulfite neurotoxicity: Direct inhibition of glutamate dehydrogenase. J. Biol. Chem. 279(41): 43035-43045.

Zimatkina, T. I., I. P. Chernikevich, S. M. Zimatkin, and R. A. Deitrich. 2000. Thiamine status in liver and brain of rats genetically selected for different sensitivity to hypnotic effect of alcohol. Alcohol. Clin. Exp. Res. 24(11): 1620-1624.