THE EFFECT OF DIETARY MANIPULATION ON THE CONTENT AND POSITIONAL DISTRIBUTION OF FATTY ACIDS INCLUDING CONJUGATED LINOLEIC ACID IN THE TISSUES OF SHEEP.

by

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Dedicated to
Amy,
I love you every morning, every day and every night
forever and ever and ever.

Abstract.

Conjugated linoleic acid (CLA) is produced in the rumen by isomerization of linoleic acid and has been shown to be anticarcinogenic. The objective of this study was to determine the effects of feeding dietary CLA to pre-ruminant lambs or linoleic acid-rich safflower oil to weaned ruminating lambs. Fat content, CLA content and fatty acid (FA) composition of diaphragm, leg, rib, subcutaneous (SC) adipose and liver tissues were determined. The triacylglycerol (TAG) fractions of the total lipid (TL) from the diaphragm, rib and SC adipose tissues were analyzed for FA composition and FA positional distribution. The phospholipid (PL) fractions of the TL were analyzed for FA composition. The positional distribution of FAs of liver PL was determined. Tissue fat content was affected by diet only in SC adipose tissue. Dietary supplementation with safflower oil increased the fat content of the subcutaneous adipose tissue whereas CLA supplementation resulted in decreased ($P<0.05$) fat content. Dietary supplementation with safflower oil increased ($P<0.05$) the CLA content of tissues by more than 200% whereas dietary supplementation with CLA did not affect CLA content. Positional distribution analysis indicated that CLA tends was localized to the sn-1/3 positions of TAG and the sn-2 position of the liver PL.

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List of Abbreviations.

BHA   butylated hydroxyanisole

BHT   butylatedhydroxytoluene

BP   benzo(a)pyrerne

$CaCl_2$ calcium chloride

$CHCl_3$ chloroform

CLA   conjugated linoleic acid

DAG   diacylglycerol

DGAT   diacylglycerol acyltransferase

DGTA   diacylglycerol transacylase

DMBA   7,12-dimthylbenz[a]anthracene

EDTA   ethylene diamine tetra acetic acid

ER   endoplasmic reticulum

FA   fatty acid

G3P sn-glycerol-3-phosphate

GC   gas chromatography

HDL   high density lipoprotein

HPLC   high performance liquid chromatography

IQ   2-amino-3-methylimidazo[4,5-f]quinoline

LA   linoleic acid

LDL	low density lipoprotein
LMW	low molecular weight fraction
LPA	lysophosphatidic acid
LPAAT	lysophosphatidate acyltransferase
MAG	monoacylglycerol
MeOH	methanol
MGAT	monoacylglycerol acyltransferase
MNU	methylnitrosourea
MUFA	monounsaturated fatty acid
N_2	nitrogen
Na_2SO_4	sodium sulfate
NaCl	sodium chloride
NL	neutral lipid
NMR	nuclear magnetic resonance
PA	phosphatidic acid
PL	phospholipid
PUFA	polyunsaturated fatty acid
SAFF	safflower oil
SC	subcutaneous
TAG	triacylglycerol
TBA	thiobarbituric acid
TL	total lipid
TLC	thin layer chromatography

TMG	tetramethylguanidine
TPA	12-o-tetradecanoylphorbol-13-acetate
UV	ultra violet
WPC	whey protein concentrate

1. Introduction.

Conjugated linoleic acid (CLA) is a unique fatty acid with health enhancing properties. CLA was first identified in the 1960s as an intermediate in the breakdown of linoleic acid by bacteria in the rumen. The discovery of CLA as an anticarcinogenic compound in the 1980s resulted in intense studies of the distribution of this fatty acid (FA) in food and research into the anticarcinogenic properties it possesses. CLA has been found in almost every food examined to greater or lesser extents but is particularly abundant in foods of ruminant origin such as milk, cheese and meat. In fact, it was initially isolated from fried ground beef as an anticarcinogenic compound by Ha and co-workers in 1987. Health conscious consumers are eating less beef and drinking less whole milk in an effort to reduce dietary saturated fat. In doing so they are depriving themselves of beneficial CLA. The health enhancing properties of CLA that have been promoted over the last ten years include cancer prevention and treatment, enhancement of the immune response, cholesterol reduction and potential as a weight reduction agent. For example, CLA does inhibit some kinds of tumorigenesis when incorporated in the diet of rodents or applied exogenously to cell culture. There has also been evidence in pigs and rodents that it can act as a fat to lean partitioning factor resulting in reduced fat deposition and enhanced growth. The positive attributes of CLA have resulted in the production and marketing of encapsulated CLA as a health supplement.

Experimental evidence shows that levels of CLA in beef and other

ruminant food products can be manipulated by dietary regime. CLA levels in milk and beef of grass fed animals are higher than those found non-grass fed animals. This is due to the higher levels of linoleic acid (LA) found in most grasses compared to grains. This can be overcome by supplementing the diets of cattle with a high LA oil thus providing the precursor of CLA to the rumen bacteria for conversion into CLA and subsequent incorporation into meat and milk fat. Although CLA is known to be present in ruminant tissues and products, little work has been done on the enhancement of CLA content as a natural product of simple dietary manipulation in meat animals or its deposition in tissues due to such manipulations. This thesis examines the effect of including pre-formed CLA in the diet of pre-ruminant lambs (*Ovis aries*) or including LA in the diet of ruminating lambs as a means of increasing the CLA content of the carcass. The positional distribution of CLA in triacylglycerols from muscle and subcutaneous fat tissue was examined under both dietary regimes in order to gain some insight into the mechanism of CLA incorporation into the acylglycerols of the ruminant tissues. The CLA content of phospholipid (PL) from these tissues was also examined. The positional distribution of CLA in the liver PL fraction of the lambs was examined for comparative purposes.

2. Literature Review.

2.1. Conjugated Linoleic Acid (CLA)

2.1.1. CLA as an Anticarcinogen

Conjugated linoleic acid (CLA) is a form of linoleic acid (LA) (C18:2, cis-9,cis-12) containing a conjugated double bond system. LA has a cis double bond at carbons 9 and 12 (Figure 1). When a single bond is present between two double bonds of the acyl chain it is known as the conjugated form. CLA has a double bond at positions 9 and 11, or 10 and 12 (Ha et al., 1987). The cis-9, trans-11 isomer of CLA is depicted in Figure 1.

CLA was first identified as an anticarcinogen in extracts from fried ground beef (Ha et al., 1987). Extracts from fried ground beef were found to contain mutagenesis modulator activity that inhibited rat liver S-9 mediated mutagenesis (Pariza et al., 1983; Pariza et al., 1985). Partially purified extracts from fried gound beef that contained this modulator activity also inhibited 7,12-dimethylbenz[a]anthracene (DMBA) initiation of mouse epidermal carcinogenesis. Through the use of semi-preparatory high performance liquid chromatography (HPLC) the anticarcinogenic fraction was purified and subsequently identified as geometric isomers of LA containing a conjugated double bond system. CLA synthesized in the laboratory was identical to the purified anticarcinogenic fraction as identified by gas chromatography (GC), ultraviolet (UV) spectroscopy and nuclear magnetic resonance (NMR) analysis (Ha et al., 1987).
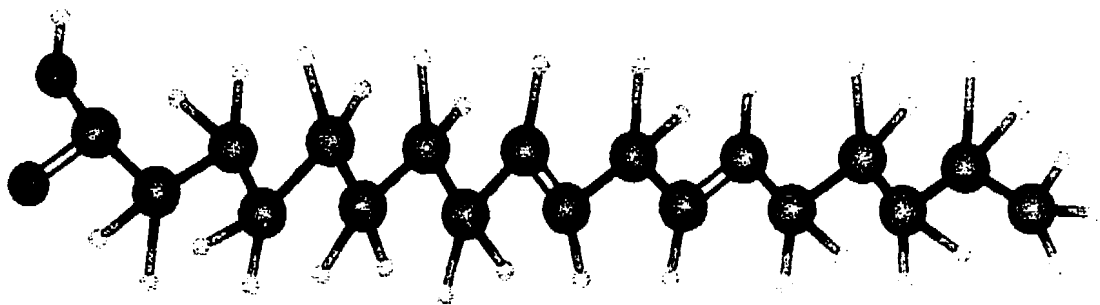
Linoleic Acid.
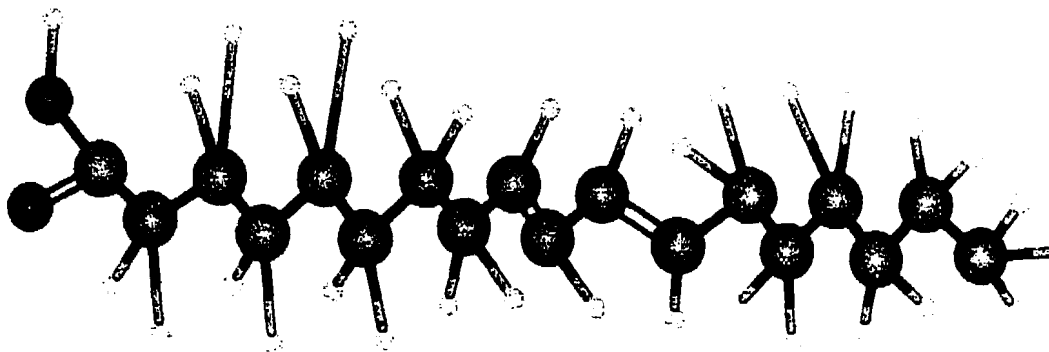
cis 9, *trans* 11 Conjugated Linoleic Acid.

Figure 1: Molecular structures of linoleic acid and *cis* 9, *trans* 11 conjugated linoleic acid. red: oxygen, dark grey: carbon, light grey: hydrogen.

There have been numerous studies into the effects of synthetic CLA on tumorigenesis when applied exogenously or included as part of the diet. When topically applied prior to DMBA treatment, CLA is also able to inhibit initiation of mouse epidermal tumors. Dietary CLA has been shown to inhibit promotion of skin tumors when included in the diet after initiation with DMBA but before promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Belury et al., 1996). Mouse stomach neoplasia induced by benzo(a)pyrene (BP) and colon carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) (a heterocyclic amine carcinogen found in grilled ground beef) in F344 rats were both inhibited by CLA administered by gavage prior to and concomitant with either BP or IQ treatment (Ha et al., 1990; Liew et al., 1995). Mammary carcinogenesis induced by DMBA was suppressed by dietary CLA, as a free acid at levels of 1.0 and 1.5% in the diet, when started 2 weeks prior to DMBA treatment (Ip et al., 1991). When 1.0% CLA was supplied as a dietary component, either as a free acid or triacylglycerol (TAG), during the post-weaning and pubertal stages of rats (21 days) and prior to administration of the tumorifgenic compound methylnitrosourea (MNU) tumorigenesis was reduced. CLA has also been shown to inhibit MNU induced tumorigenesis when administered after MNU treatment, but a continuous intake of CLA was necessary for maximal and lasting inhibition (Ip et al., 1995). Additionally, dietary CLA has been shown to have an hypocholesterolemic and antiatherogenic effect in rabbits (Lee et al, 1994).

2.1.2. CLA as an Antioxidant

The antioxidant activity of CLA has been offered as a possible explanation of the anticarcinogenic and antiatherosclerotic properties of CLA. The antioxidant potential of CLA was compared with known antioxidants using the thiocyanate method. In this test using free fatty acids (FAs) in a phosphate buffer and ethanol mixture the degree of LA oxidation was determined by the addition of ferrous ammonium sulfate and thiocyanate then followed by absorbance determinations at 480nm. CLA was found to be more powerful than α-tocopherol and comparable to butylated hydroxytoluene (BHT) as an antioxidant *in vitro* (Ha *et al*, 1990). Antioxidant activity of CLA *in vivo* was reported by Ip *et al* (1991). Mammary and liver tissue from rats fed either CLA, vitamin E or butylated hydroxyanisole (BHA) were tested for thiobarbituric-acid (TBA) reactive substances that are presumably lipid peroxidation products. Results of this study showed that CLA decreased the amount of TBA reactive substances detected in mammary tissue but had no effect on the amount present in liver tissue. Subsequent work by van den Berg *et al* (1995) disputed the claim of antioxidant activity of CLA measured by these methods, stating that the conditions of the thiocyanate method are very dissimilar to biological systems and that the TBA assay is known to be indirect and not specific. They indicated in their tests, using a model system of phospholipid (PL) membranes subjected to various types of oxidative stress, that CLA was insufficiently reactive toward radicals in comparison to α-tocopherol and BHT for it's mode of action to be that of a radical scavenger

2.1.3. CLA as a Growth Factor

Chronic CLA feeding has been shown to have no effect on animal growth rate (Ip et al, 1991). Observations that CLA was able to reduce catabolism of skeletal muscles after immune stimulation in mice, rats and chickens (Cooke et al., 1993; Miller et al., 1994) led to the hypothesis that CLA may function as a growth factor by reducing catabolism of skeletal muscle which reduces the energy available for growth, thus functioning as an energy partitioning agent. While consumption of CLA had no effect on food intake or weight gain of rat dams during gestation, dietary CLA at levels of 0.5% for dams resulted in significantly higher pup weight at ten days of age compared to pups of dams receiving control diets or 0.25% CLA during gestation and lactation. Pups from dams that received 0.5% CLA only during lactation were of intermediate weight (Chin et al., 1994b). CLA (0.5%) in the diets of male mice for 32 days post weaning and female mice for 28 days post-weaning resulted in a reduction in percent body fat by 57% and 60% respectively (Park et al., 1997). Whole body weights of control and treatment groups showed no significant differences (Park et al., 1997). In the same study, it was determined that percent protein and percent water in the treatment animals was significantly enhanced compared to controls.

The desire of the meat industry to produce animals with improved lean production efficiency has prompted research into the value of feeding CLA to meat animals. Feeding a diet containing CLA to pigs has produced animals with an overall increased gain to feed ratio and lean tissue depostion and reduced

subcutaneous fat (SC) deposition (Dugan *et al.*, 1997; Ostowska *et al.*, 1999).

2.1.4. Action and Metabolism of CLA

The actual mechanism of CLA action is poorly understood. CLA is relatively stable (does not oxidize) when exposed to air at room temperature (Ha *et al.*, 1987) compared to the level of oxidation of LA. CLA also exhibits antioxidant activity, inhibiting peroxide formation that is higher by 90% or more of the levels inhibited by α-tocopherol (vitamin E) and to levels similar to that of BHT when assayed by the thiocyanate method at a molar ratio of 1 part CLA to 100 parts LA *in vitro* (Ha *et al.*, 1990). Antioxidant activity, as evaluated by the TBA method, followed the effect of CLA feeding ranging from 0.25% to 1.5% in the diet. The amount of TBA reactive substances (lipoperoxidation products) detected in liver did not change but there was a significant decrease in these substances in the mammary gland (Ip *et al.*, 1991).

Despite the previous evidence that CLA can act as an antioxidant *in vivo* (Ip *et al.*, 1991) and *in vitro* (Ha *et al.*, 1990), investigations into the mechanism of CLA's anticarcinogenic effect provide evidence that CLA actually increases lipid peroxidation in some cultured cell lines. Human lung carcinoma cell lines, A4-27, SK-LU-1 and A549 showed a dose and time dependent growth inhibition with CLA. Addition of α-tocopherol (a known antioxidant) to the growth medium resulted in partial growth recovery of these cell lines. Levels of lipid peroxidation products, as identified by the TBA method, were several fold higher in the CLA cultures than the control cultures (Schonberg and Krokan, 1995).

CLA also appeared to inhibit cell growth in tissue culture. Schultz *et al.*

(1992) found a dose and time dependent inhibition of cell growth by CLA in the human MCF-7 breast cancer cell line that was more consistent than inhibition by LA at a bovine serum albumin concentration of 2.3 mg/ml. desBordes and Lea (1995) found that 100 mM LA inhibited thymidine incorporation more than CLA at the same concentration in the MCF-7 cell line, the 7800NJ rat heptoma cell line and T47D human breast cancer cells. CLA and LA, however exhibited similar inhibitory effects at concentrations of 500 mM. Increasing the concentration of albumin from 1 to 38 mg/ml greatly diminished the inhibitory effects of both CLA and LA at 500mM concentrations.

In tests to determine if CLA could protect membranes composed of 1-palmitoyl-2-linoleoyl-phosphatidylcholine from oxidation, CLA was less effective than α-tocopherol and BHT as a radical scavenging antioxidant (van den Berg *et al.*, 1995). Tests of CLA's stability during the same study indicated that CLA was more susceptible to oxidation than LA. This was contrary to the results of Ha *et al.* (1987). Four furan FAs have been identified as oxidation products of CLA by GC analysis. These are 8,11-epoxy-8,10-octadecadienoic acid ($F_{8,11}$); 9,12-epoxy-9,11-octadecadienoic ($F_{9,12}$); 10,13-epoxy-10,12-octadecadienoic ($F_{10,13}$) and 11,14-epoxy-11,13-octadecadienoic ($F_{11,14}$) (Yurawecz *et al.*, 1995). In determining the oxidation products of CLA, Yurawecz *et al.* (1995) found that CLA was very stable in methanol solutions containing 10% or less of water. Greater yields of furan FAs as oxidation products were associated with a more efficient suspension of CLA. Increasing water to 50% of the solution produced the maximum oxidation of CLA.

Other suggested modes of action of CLA's anticarcinogenic effects may be due it's ability to inhibit protein and nucleotide biosynthesis (Schultz *et al.*, 1992) prostaglandin E synthesis and ornithine decarboxylase activity (Liu and Belury, 1997); these events are strongly linked to skin tumor promotion.

Conjugated forms of C18:3 and C20:3 have been detected in rat liver and lamb liver which also contained conjugated forms of C20:4 (Banni *et al.*, 1996). These FAs were further characterized and identified as C20:3 Δ8,12,14; C20:4 Δ5,8,12,14; C20:4 Δ5,8,11,13 (Sebedio *et al.*, 1997). These FAs likely arose from elongation and desaturation of C18:2 Δ10,12 and C18:2 Δ9,11 (Sebedio *et al.*, 1997).

2.1.5. CLA in Food

Chin *et al.* (1992) conducted an extensive survey of the presence and concentrations of CLA in various foodstuffs. This study indicated that the principal dietary sources of CLA were meat and dairy products. Meat from ruminants (beef, lamb) generally contained more CLA, ranging from 2.7 to 5.6 mg CLA/g fat, than meat from nonruminants, such as chicken, pork and seafood, which had CLA concentrations of 0.3 to 0.9 mg CLA/g fat. Dairy products also showed high levels of CLA. Cow's milk contained 5.5 mg CLA/g fat. Cheeses contained from 2.9 to 7.1 mg CLA/g fat and yogurt ranging from 1.7 to 4.8 mg CLA/g fat. Oils from plants had a lower CLA content ranging from 0.1 to 0.7mg CLA/g fat whereas beef tallow had a CLA content of 2.6 mg CLA/g fat.

The early work of Ha *et al* (1987 and 1989) indicated that cooking of ground beef and the processing of cheese led to an increase in concentrations of

CLA. The postulated mechanism for this production of CLA was through the presence of a LA radical which interacted with hydrogen to form a double bond system.

The food survey of Chin *et al.* (1992) indicated that, in general, processing did not alter total CLA content on a mg CLA/g fat basis and that total CLA in canned foods was similar to that of comparable unprocessed foods. Similarly work of Shantha *et al* (1994) indicated that different cooking methods such as frying, broiling, baking or microwaving did not result in any significant changes in the CLA content of ground beef when compared on a mg CLA/g fat basis. Cooking method and degree of doneness can affect the concentration of dietary CLA from beef products since cooking methods can influence total fat content.

The varying concentrations of CLA in cheeses may be related to the CLA content of the starting product, milk. The CLA content of milk has been shown to be variable, ranging from 2.4 to 21.8 mg CLA/g fat (Riel, 1963). It was hypothesized that this may occur because of seasonal variations in pasture feeding practices (Shantha *et al*, 1995; Riel, 1963; Parodi, 1977) and diet (Chin *et al.*, 1994a; Jiang *et al.*, 1996; Kelley *et al.*, 1998). There is also evidence to indicate, however, that addition of whey protein concentrate (WPC) to processed cheese results in an increased CLA content, specifically addition of the low molecular weight fraction (LMW) of WPC. The CLA content in the processed cheese increased by 1.73mg/g fat. The maximum amount CLA content could have increased based on fat content of WPC alone would have been 0.016 mg/g fat. The ability of the WPC and whey LMW fraction to increase the formation of

CLA could have been due to interactions between components of the whey LMW fraction and LA radicals (Shantha *et al*, 1992).

Processing of cheeses at temperatures greater than 80°C also increased the formation of CLA when the processing was done under atmospheric conditions. Processing under nitrogen has shown no such increases indicating that both temperature and the presence of air play a role in CLA formation. The ability of increased temperature and/or the presence of air may result in the formation of oxygen radicals and subsequently LA radicals, thus increasing CLA concentrations upon addition of hydrogen from protein sources to form the conjugated double bond system (Shantha *et al*, 1992).

Further work by Shantha *et al*. (1995) on other dairy products indicated that total CLA concentrations in salted and unsalted butter increased approximately 1.3-fold from that of the starting material and that nonfat yogurt had more CLA than it's starting material. Conversely, work carried out by Werner *et al* (1992) suggests that the use of different starter cultures, processing conditions and aging periods had a negligible effect on total CLA concentration. Work by Lin *et al* (1995) concluded that activity of microbial metabolic reactions may be related to the differences in the CLA content of these products as well as the processing conditions.

2.2. The Rumen

2.2.1. Anatomy of the Rumen

The ruminant stomach is a four-chambered organ that functions to digest
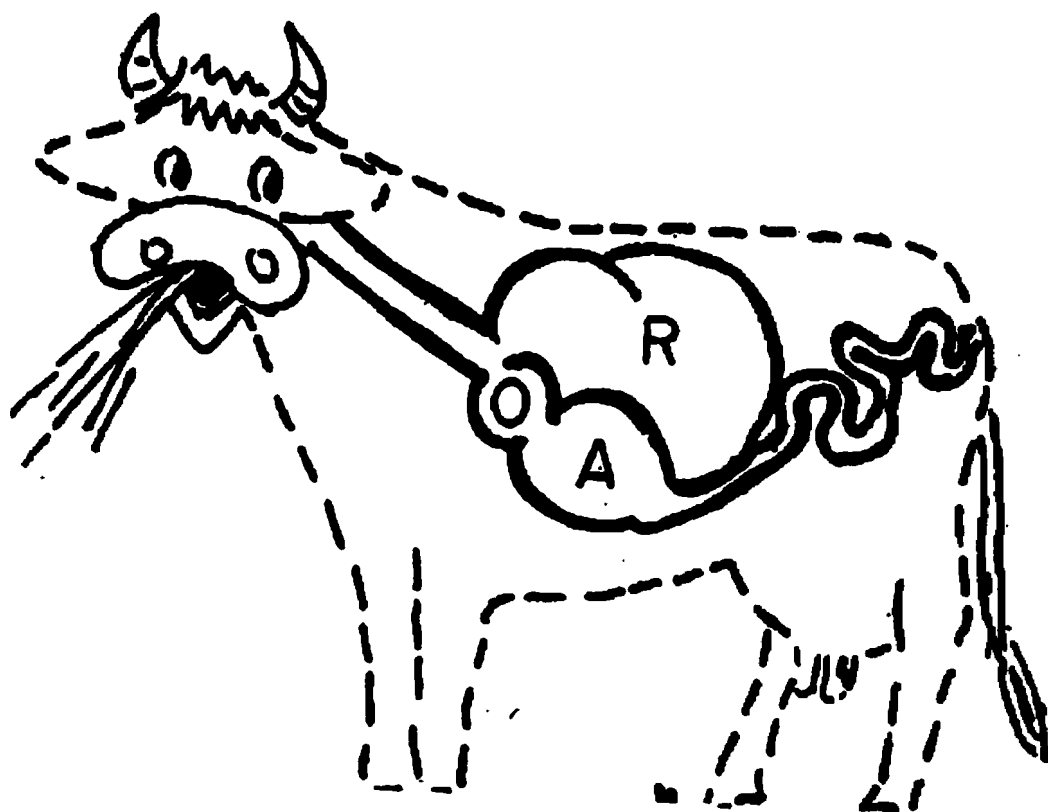
Figure 2. Cartoon of cow showing the relative positions of the rumen (R), omasum (O), and abomasum (A) in the ruminant digestive system.

the grains and forages eaten by ruminant animals (Van Soest, 1994). The four chambers are the rumen, reticulum (often referred to as the reticulorumen) omasum and abomasum (Figure 2). In young ruminants the rumen is undeveloped and suckled milk is bypassed to the abomasum due to closure of the esophageal groove during sucking and swallowing. Rumen development is stimulated by ingestion of solid feed and inoculation by rumen bacteria through feed and feces and contact with other animals.

2.2.2. Digestion

Food is initially masticated and mixed with saliva sufficiently to form a bolus that is swallowed and enters the anterior portion of the rumen (Van Soest, 1994). The rumen represents a microbial population that facilitates the celluolytic, proteolytic and lipolytic breakdown of feedstuffs. The reticulorumen contracts and relaxes to mix the ingesta. Integrated with this process is rumination. Rumination is the regurgitation of ingesta to be remasticated to further reduce particle size and extract water-soluble contents with saliva from the initial feeding event. This integrated process allows for the selective turnover of indigestible food which can be regurgitated and masticated as many times as necessary. Fine particles and liquid will flow from the reticulorumen into the omasum through the reticulo-omasal orifice. The omasum functions to absorb water and nutrients with leaf like structures which can also block passage of any large particles of digesta, and to pump the digesta from the reticulum to the abomasum through the omasal canal and omasal-abomasal orifice. The

abomasum is the true stomach of the ruminant where gastric juices are secreted

and peptic digestion takes place. This includes the digestion of microbial cells

that arrive from the rumen by pepsin and lysozyme secretions. The now acidic

digesta continue their flow out of the abomasum through the pylorus and into the

duodenum where mixing with both biliary and pancreatic secretions facilitate

homogenization of fats and hydrolysis of starch, proteins and TAGs. Most

soluble starches and TAGs are broken down in the rumen and do not reach this

stage of digestion. Nutrient absorption takes place in the small intestine. In the

large intestine, the colon and caecum function to absorb electrolytes and water

and break down any slow digesting feed that escapes from the reticulorumen.

2.2.3. Lipid Metabolism in the Rumen: Lipolysis and Biohydrogenation

There are two types of FAs. Saturated FAs have only single bonds in the

acyl chain. Unsaturated FAs have at least one double bond present in the acyl

chain. Unsaturated FAs can be further subdivided into *cis* and *trans* forms. The

position of this bond affects the conformation of the individual FA. A *cis* bond will

result in a bend in the acyl chain whereas a *trans* bond will result in a straight

chain form like that of a saturated FA.

Saturated FAs are considered undesirable and have been linked to

coronary heart disease and cancer. Saturated FAs produce harder fats due to

the ease of 'stacking' of the straight chain form. There are also possible risks

perceived in the consumption of high levels of the unsaturated *trans* FAs due to

their similarity in structure to saturated FAs. TAGs, containing unsaturated fatty

acyl moeities, found primarily in plants are considered more desirable in the

human diet as they are softer.

Hydrogenation is a process by which unsaturated FAs are converted to saturated FAs. This occurs during processing of vegetable oils to produce margarine that is soft but not liquid. In ruminant animals this is a natural process carried out by rumen microbes, and referred to as biohydrogenation. The unsaturated fats, LA and linolenic acid (C18:2 and C18:3) found in grains and forages respectively are bio-hydrogenated in a multi-step process from to the completely saturated form, stearic acid (C18:0). The *trans* form of (t-11)18:1 is an intermediate of this biohydrogenation process as is the c9,t11 isomer of LA known as CLA.

Increasing intake of *trans* FAs at the expense of *cis* fats has been shown to increase plasma low density lipoprotein (LDL) cholesterol levels, raise lipoprotein (a) (Lp(a)) levels and lower high density lipoprotein (HDL) cholesterol levels. These results indicated that increased *trans* FA intake is unfavourable in view of increased cardiovascular disease risk (Zock and Katan, 1997, Precht and Molkentin, 1995). *Trans* FAs have not been shown, however, to increase cancer risk in animal model studies (Ip *et al*, 1991) when increased at the expense of *cis* forms.

Once lipids enter the rumen there are two important microbial transformations that take place; lipolysis of esterified plant lipids is followed by biohydrogenation of the free FAs produced to reduce the number of double bonds (Jenkins, 1993). Biohydrogenation in the rumen ultimately results in formation of saturated FAs from unsaturated FAs. Intermediate forms of poly-

and monounsaturates may also be incorporated into tissues. The biohydrogenation process is facilitated by the rumen bacterial and protozoal population (Girard and Hawke, 1978). Rumen microorganisms such as *Anaerovibrio lipolytica* and *Butyrivibrio fibrisolvens* produce lipases, phopholipases and galactolipases. Acylglycerols, galactolipids and phopholipids are hydrolyzed to free FAs and galactose or glycerol. The galactose and glycerol are fermented in the rumen to volatile FAs with propionic acid being the final product. Unsaturated FAs are rapidly hydrolyzed by rumen microorganisms to more saturated end products. Generally the acids are converted to a mores stable *trans* form and double bonds are distributed in many positions along the acyl chain.

The initial steps in the biohydrogenation of unsaturated FAs are carried out by the bacterium, *B. fibrosolvens*. An isomerase catalyzes the formation of a *trans* double bond only when the substrate contains a c9,c12 double bond system and has a free carboxyl group (Garton, 1977). c9,t11, c15 octadecatrienoic acid is produced from its parent form of c9,c12,c15 octadecatrienoic acid (linolenic acid) and c9,t11 octadecadienoic acid from c9,c12 octadecadienoic acid (LA). Enzymes of *B. fibrosolvens* continue to catalyze the conversion of the conjugated forms to the t11 octadecenoic form (vaccenic acid). Other rumen bacteria are responsible for the final conversion to stearic acid, the fully saturated form of C18:0 (Kepler *et al.*, 1966). Complete hydrogenation to a saturated form depends on conditions in the rumen. It is promoted by the presence of cell free ruminal fluid and feed particles, and

inhibited by large amounts of LA (Jenkins, 1993). Because the *trans* form is more difficult to hydrogenate, there is an accumulation of *trans* forms relative to *cis* forms.

Lipids entering the small intestine are 70% saturated non-esterified FAs, which are of both dietary and microbial origin (Van Soest, 1994). The contribution of the microbial population to the lipids entering the small intestine are long chain FAs taken up by bacteria thus escaping biohydrogenation, and, FAs that are synthesized *do novo* (Van Soest, 1994). Some C16:1 and C18:1 are produced by an anaerobic monounsaturated FA synthesis pathway or by action of an anaerobic desaturase on stearic acid (Jenkins, 1993).

2.3. Glycerolipid Biosynthesis

Glycerolipids are produced by esterification of FAs to a glycerol backbone through the action of the enzymes of the *sn*-glycerol-3-phosphate (G3P) pathway, monoacylglycerol (MAG) pathway, or by the action of diacylglycerol transacylase (DGTA) (Lehner and Kuksis, 1996). All of these pathways are associated with the microsomal fraction.

The G3P pathway is associated with rough endoplasmic reticulum (ER) in liver and adipose tissue cells (Lehner and Kuksis, 1996). The stepwise esterification of FA moieties from acyl-CoA to the glycerol backbone produces lysophosphatidic acid (LPA) by the catalytic action of *sn*-glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15) acting on G3P, or by the acylation of dihydroxyacetone phosphate which involves the catalytic action of the enzyme

dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42) followed by a reduction reaction. Dihydroxyacetone phosphate is produced during glycolysis. Phosphatidic acid (PA) is produced by the esterification of a FA to sn-2 of the LPA by lysophosphatidate acyltransferase (LPAAT) (EC 3.1.3.20). The PA is then dephosphorylated by the catalytic action of phosphatidate phosphohydrolase (PA phophatase) (EC 3.1.3.4) to produce an sn-1,2-diacylglycerol (DAG). A third FA is attached to the sn-3 position of DAG by the catalytic action of diacylglycerol acyltransferase (DGAT) (EC 2.3.1.20) to form TAG. Alternatively the sn-1,2-DAG is used in other pathways for the synthesis of PLs such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine.

The MAG pathway is associated with the smooth ER in intestine cells (Lehner and Kuksis, 1996). The MAG pathway produces a DAG through the acylation of 2-MAGs, produced in the intestine by the action of pancreatic lipase, with the enzyme monoacylglycerol acyltransferase (MGAT) (EC 2.3.1.22). The DAG can then be utilized in the same way as those produced by the G3P pathway.

Diacylglycerol transacylase (DGTA) catalyzes the synthesis of TAG from two DAG molecules in the absence of acyl-CoA by transferring an acyl chain form one DAG and leaving a 2-MAG as its other product. The enzyme was first purified from microvillus cells of rat intestine (Lehner and Kuksis, 1996). In non-ruminant systems, the liver and intestine are involved in the synthesis and secretion of TAGs and adipose tissue is responsible for TAG storage and release

of FAs (Lehner and Kuksis, 1996). In the bovine, adipose tissue has a higher

level of glycerolipid and FA biosynthesis than the liver (Wilson *et al.*, 1992 ; Ingle

et al., 1972). Approximately 90% of lipid synthesis takes place in adipose tissue

whereas the liver accounts for only 5% (Van Soest, 1994). The MAG pathway

has not been studied in adipocytes of meat animals (Rule, 1995), but has been

shown to be active in neonate sheep intestine and the ability to use this pathway

is retained in adult sheep (Cunningham and Leat, 1969). The role of DGTA in

animal TAG synthesis is in need of further investigation. Preliminary results have

suggested that there might be some DGTA activity in bovine adipose and muscle

tissues (R. J. Weselake, unpublished results).

2.4. Effect of Dietary Lipids on FA Composition of Lipid Classes and Positional Distribution of FAs in Glycerolipids

Dietary lipids that are broken down during ruminal lipolysis and

biohydrogenation can be used by rumen bacteria as substrates for cellular

growth. The generally saturated free FAs that are created in the rumen are

absorbed and transported to other tissues as plasma lipoproteins. The chemical

composition and the rate of secretion of lipoproteins are among the main factors

that control lipid utilization by the tissues (Bauchart, 1993). Chylomicrons are

synthesized by the ruminant intestine and transport dietary FAs to the tissues for

fat storage or milk production. Increasing dietary fat stimulates secretion of

chylomicrons or dietary polyunsaturated fat compared with saturated FAs. The

presence of saturated FAs leads to secretion of LDL molecules by the intestinal

mucosal cells.

Dietary fat is rapidly metabolized in the ruminant to a saturated form. Dietary manipulation of fat in order to result in an alteration of FA composition of tissues and milk is generally attempted through the use of what is known as protected fat. This is a dietary lipid supplement treated in such a way that fat is not metabolized in the rumen and passes to the small intestine as intact TAG. Pancreatic lipases then catalyze the degradation of the protected TAG to release FAs that have not been modified in the rumen. In this way polyunsaturated or other specific FAs can be absorbed. Dietary supplementation with different dietary oils has been shown to increase the CLA content of milk (Kelly *et al.*, 1998). CLA infused directly into the abomasum at a rate of 150g day^{-1} to by-pass the rumen fermentation increased the milk fat CLA content by 10-fold while reducing the fat content of the milk by 50%. (Chouinard *et al.*, 1999)

In non-ruminants, the FAs are not microbially altered before being incorporated into lipid biosynthesis pathways. Studies involving chickens, pigs and humans have addressed the incorporation of dietary CLA into tissues. Egg yolk lipids analyzed after feeding hens CLA determined that CLA content of the egg yolk lipid was proportional to the level of CLA in the diet. More CLA was incorporated into the TAG fraction of the TL than the PL fraction (Du *et al.*, 1999), and concentrations of 18:1, 18:2, 18:3, 20:4 and 22:6 were decreased (Chamruspollert *et al.*, 1999). When pigs were fed CLA of known isomer composition the isomer distribution detected in backfat and omental fat TL, liver TAG, free FAs and cholesterol esters showed a similar pattern to that of the diet

(Kramer *et al.*, 1998).

Positional distribution refers to the organization of FAs esterified to the glycerol backbone of glycerolipids. The positions are numbered *sn*-1, *sn*-2, and *sn*-3 in Fisher projection (Figure 3) such that the FA on the middle carbon is on the left hand side and is considered to be in the plane of the page and the carbon is deignated *sn*-2. The top carbon is numbered *sn*-1 and the bottom carbon is numbered *sn*-3. The distribution of FAs on the glycerol backbone of animal fats is non-random and the positional distribution of FAs on the glycerol backbone of lipids is influenced by the length of the acyl chain and its degree of unsaturation. Shorter and more unsaturated FAs tend to occupy position 2 (Brockerhoff *et al*, 1966). Unusual FAs, along with foreign FAs, usually end up in the *sn*-3 position of the TAG molecules (Lehner and Kuksis, 1996)

Positional distribution of FAs can be influenced by diet. Sheep fed protected sunflower seed oil that bypasses rumen biohydrogenation showed increased incorporation of 18:2 in the *sn*-1 and *sn*-2 postions of TAG (Hawke *et al*, 1977; Mills *et al*,1975). Protection from biohydrogenation results in a higher percentage of 18:2 available for incorporation into glycerolipids. A recent study by Smith *et al.* (1998) showed positional distribution changes in cattle fed various monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) diets. Positional distribution of FAs on the glycerol backbone is thought to influence physical characteristics of the fat. Fats that are high in 18:0 in the 1 and 3 positions are harder and are poorly absorbed which may explain why they are hypocholesterolemic.

H
|
H — C — O — FA   *Position sn-1*
|
FA — O ◀ C ▶ H   *Position sn-2*
|
H — C — O — FA   *Position sn-3*
|
H
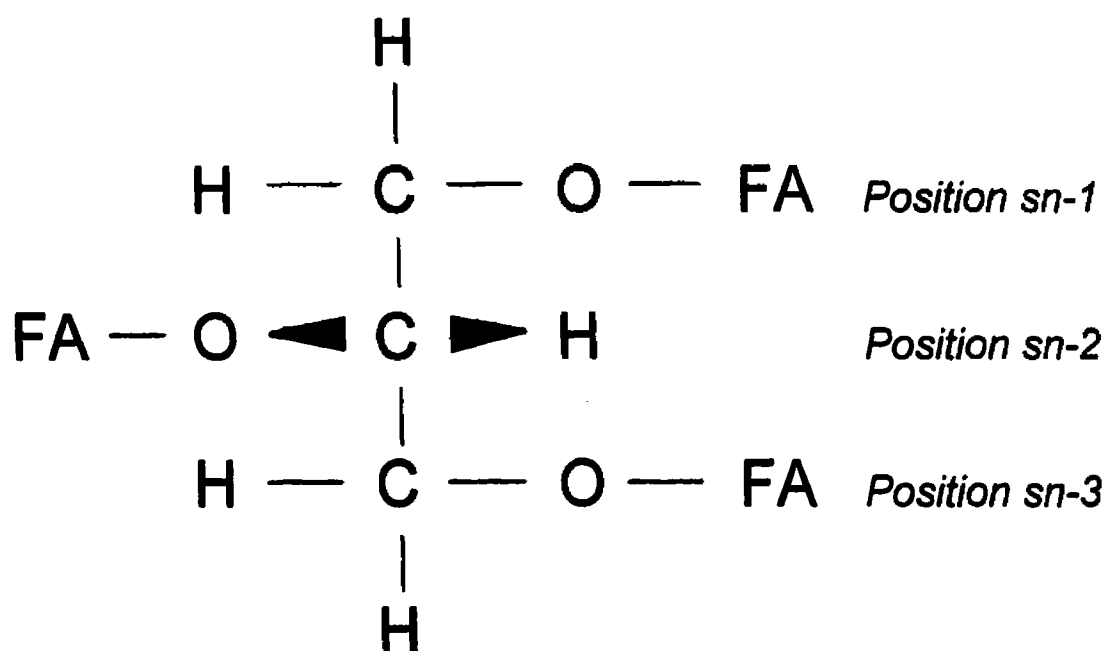
Figure 3. Positional distribution and stereochemical numbering scheme on a Fisher projection model of a triacylglycerol molecule.

2.5. Hypothesis and Experimental Strategy

The questions I wanted to answer in this thesis were whether dietary supplementation with CLA to pre-ruminant lambs, or LA rich oil (safflower oil) to weaned ruminating lambs, had any effect on lipid deposition in tissue, FA composition of tissue, and the positional distribution of FAs in TAG and PL. These studies may provide insight into possible mechanisms for incorporation of CLA into acylglycerols of animal tissues.

Supplementation of ruminant diets with CLA, as conducted by Dugan *et al.* (1997) for non-ruminants is not possible because CLA would be rapidly hydrogenated in the rumen to stearic acid. In order to avoid this biohydrogenation, dietary supplementation with CLA has to occur when the animals are not ruminating. Pre-ruminant animals exhibit closure of the esophageal groove, a conditioned reflex, when consuming fluid meals up till weaning (Roy and Stobo, 1975) and are essentially similar to non-ruminants. In such a situation fluid meals pass directly from the mouth into the abomasun, avoiding the rumen and the possibility of biohydrogenation, thereby allowing the animal to absorb the dietary CLA unmodified in the intestine.

For this study four four-week-old pre-ruminant lambs were supplemented with CLA in milk replacer, and four seven-week-old weaned ruminating lambs were supplemented with LA rich oil in pelleted feed. Five, lambs were carried through both feeding phases as controls with no dietary supplement. At slaughter tissue samples were taken for analysis. Data obtained from feeding pre-ruminant animals with CLA, and those receiving LA rich oil supplemented

feed as weaned animals was compared with control animals receiving no supplements.

The work described in the following paper was presented by my co-supervisor, Dr. Z. Mir, at the *Canadian Society for Animal Science* annual meeting in Montreal in 1998. I was unable to prepare and present the material in its entirety due to timing and funding constraints. I did, however, run the feeding trial and tissue sampling aspects of the experiment and I was also responsible for establishing the lipid extraction, methylation, gas chromatography and statistical analysis protocols. Mr. M. L. Rushfeldt assisted in the analysis of the TL extracted from the sampled tissues by carrying out these established protocols while my focus changed to establish positional distribution analysis protocols used in the further characterization of the PL and TAG fractions of the TL from samples procured from animals in the trial. The following manuscript on this work has been published in *Small Ruminant Research* as:

Effect of dietary supplementation with either conjugated linoleic acid (CLA) or linoliec acid rich oil on the CLA content of lamb tissues. Z. Mir, M. L. Rushfeldt, P. S. Mir, L. J. Paterson and R. J. Weselake. Small Ruminant Research, Volume 36, Issue 1, April 2000, pages 25-31.

3. Effect of Dietary Supplementation with either Conjugated Linoleic Acid (CLA) or Linoleic Acid Rich Oil on the CLA Content of Lamb Tissues.

3.1. Abstract

Conjugated linoleic acid (CLA) is produced in the rumen by isomerization of linoleic acid (LA) and has been shown to be a potent anticarcinogen in animal model studies. The objective of this study was to compare the relative increase in the CLA content of lamb tissues by dietary CLA supplementation (0.33 g/d for 21 days prior to weaning) to milk-replacer of pre-ruminant lambs or by feeding LA rich oil (Safflower oil, 6% DM- SAFF) to weaned ruminating lambs with that of lambs receiving unsupplemented milk replacer and pelleted feed. Thirteen lambs were randomized to three dietary treatments (CLA - 4, SAFF - 4 and Control - 5) and fed the pelleted diet for 80 days after weaning. Lambs were slaughtered at an average weight of 45 kg and tissue samples were procured from pars costalis diaphragmatis, leg, rib, subcutaneous adipose and liver for determination of fat and CLA content and fatty acid composition. Dietary supplementation with safflower oil increased fat content of subcutaneous adipose tissue only, but the CLA content of all the tissues was increased (P < 0.05) by more than 200%. Dietary safflower oil increased (P < 0.05) C18:2 in all tissues and C16:0 in the diaphragm and decreased (P < 0.05) C18:1 and C18:3 content in all tissues. Supplementation of the diet with preformed CLA prior to weaning decreased (P < 0.05) fat content of the adipose tissue with decreases occurring in C18:0

relative to animals receiving the unsupplemented diet However, tissue CLA

content was not affected by provision of dietary CLA to preruminant lambs.

Results indicated that supplementation of lamb feedlot diets with a source of LA

was a successful method of increasing CLA content of tissues.

3.2. Introduction

Conjugated linoleic acids (CLA) are produced by bioconversion of LA by

the bacterium *Butyrivibrio fibrosolvens* in the rumen (Kepler et al. 1966) and are

intermediary metabolites in the production of stearic acid. Currently, CLA are

defined as a combination of several positional and geometric isomers with double

bonds predominantly at the 9 and 11, 10 and 12 or 11 and 13 carbon atoms, with

various combinations of *cis* and *trans* configuration at each double bond.

Although CLA represent a relatively minor component of the total fatty acid

composition of foods, CLA exhibit chemoprotective properties against

carcinogens when consumed at very low levels (<1% of the diet; Ip, 1997 or 3.5

g/d Ha et al., 1989). These values were extrapolated from rat studies. Recently

Knekt et al.(1996)reported reductions in incidence of breast cancer in women

consuming 55mg CLA /d from milk. In general, meat and dairy products from

ruminants contain more CLA than products from nonruminants (Fogerty et al.,

1988; Jiang et al., 1996). The concentration of CLA in beef ranges from 1.7 to

8.5 mg CLA/g fat and is dependent on the nature of feed fed to the animals

(Shantha et al., 1994; Mir et al., 1999). Canola oil or full-fat rapeseed

supplementation of diets fed to goats or dairy cows, respectively, increased CLA

content in milk, ranged from 10.35 (control) to 32.05 and 3.91 (unsupplemented) to 7.89 mg per g of lipid (Mir et al., 2000; Stanton et al., 1997). To our knowledge, effects of dietary oil supplementation on CLA content in ruminant muscle, adipose and liver tissues have not been reported previously.

Supplementation of ruminant diets with CLA, as conducted by Dugan et al. (1997) for nonruminants, is not possible because CLA would be rapidly hydrogenated in the rumen to stearic acid. In order to avoid this biohydrogenation, dietary supplementation with CLA has to occur when the animals are not ruminating. Preruminant animals exhibit closure of the esophageal groove, a conditioned reflex, when consuming fluid meals up till weaning (Roy and Stobo,1975).and are essentially similar to non-ruminants In such a situation fluid meals pass directly from the mouth into the abomasum, avoiding the rumen and the possibility of biohydrogenation, thereby allowing the animal to absorb the dietary CLA unmodified in the intestine.

The objective of this study was to compare the relative increase in the CLA content of lamb tissues by dietary CLA supplementation to milk-replacer of pre-ruminant lambs or by feeding LA rich oil (safflower oil) to weaned ruminating lambs with that of lambs receiving unsupplemented milk replacer and pelleted feed.

3.3. Materials and Methods

3.3.1. Lambs and Diets

Thirteen, four week old crossbred lambs (avg. wt. 12.5 ±3.43 kg) were

removed from ewes and placed in two pens with free access to milk replacer

from an artificial ewe feeding apparatus for 21 days. The lambs had free access

to a pelleted creep feed containing barley grain, alfalfa, and soybean meal. Each

of the lambs in the control treatment (5 lambs) and those designated to receive

the safflower oil (SAFF; 4 lambs) upon weaning were given 50 mL of milk

replacer containing 5 mL of olive oil (carrier), daily by bottle. Lambs in the CLA

treatment (4 lambs) received 50 mL of milk replacer containing 0.33 g CLA

(Pharmanutrients, Lake Bluff IL, USA) dissolved in 5 mL of olive oil daily.

After 21 days, the lambs (avg. wt. 18.9 ± 3.38 kg) were weaned off the

artificial ewe and, housed in individual pens and provided with a pelleted diet ad

libitum. The lambs in the control treatment and those that received CLA

supplemented milk-replacer, were provided with the pelleted diet, while the lambs

in the SAFF treatment received the pelleted diet supplemented with 6% safflower

oil on a dry matter basis. Safflower oil purchased from a commercial source was

guaranteed to contain 78% linoleic acid and Chin et al (1992) reported that

commercial safflower oil contains 0.7 mg CLA/g fat. The ingredient composition

of the pelleted diet was: barley, 56.2%; dehydrated alfalfa, 29.5%; soybean meal,

9.0%; molasses, 2.0%; sheep mineral, 1.05%; maxi-pel, 1.0%; $CaPO_4$, 0.5%;

NH_4Cl, 0.5%; dicalcium phosphate, 0.2%; vitamin A (10 million IU kg^{-1}), vitamin D

(1 million IU kg^{-1}), and vitamin E (100 000 IU kg^{-1}), 0.0333%; and deccox premix,

0.0181%. The crude protein of this diet was 16.6%. Lambs were fed for 80±1.8

days on this diet. The average weight at slaughter was 45.9 ± 2.9kg.

3.3.2. Tissue Samples

At the time of slaughter, samples of the diaphragm, liver and adipose tissue of each animal were obtained. A leg roast and double chop from the 12th and 13th ribs were later sampled from each animal at the time of cutting. All samples were obtained from the same half of the carcass. All of the samples were stored at -40°C until ready for analysis. Samples were thawed at 4°C and subsamples were taken for dry matter analysis and ground with a hand grinder until they were completely blended, and then were refrozen at -20°C.

3.3.3. Lipid Extraction and Fatty Acid Analysis

The Folch method (Folch et al 1957) was used for lipid extraction. Approximately 500 mg of frozen meat samples were weighed into a test tube, and 1 mL of chloroform/methanol (2:1 v/v) mixture was added. The sample was homogenized in a ground glass tissue grinder, for 30 sec at 4°C. The homogenizer was rinsed two times in 2 mL of the same chloroform/methanol mixture (total 4 mL), and the rinse fluid was combined with the homogenate. The tube was flushed with nitrogen and spun at 3000 rpm (1250 x g) for 30 min at room temperature. The supernatant was quantitatively transferred to a clean test tube and 400 FL of 0.29% NaCl solution were added. The tube was flushed with nitrogen and placed on a test tube rocker for 10 min, after which it was centrifuged at 3000 rpm (1250 x g) for 30 min at room temperature. The chloroform layer was transferred to pre-weighed vials equipped with teflon-lined caps, flushed with nitrogen and stored at -20°C until required for methylation.

The chloroform was evaporated under nitrogen while the vials were placed

in a 40°C water bath, and the total amount of lipid extracted was determined

gravimetrically. A known amount of extracted lipid was derivatized using

tetramethylguanidine (TMG) and methanol, as described by Shantha et al.

(1993), and the fatty acids in the samples were separated by gas

chromatography as described by Mir et al. (1998b).

3.3.4. Statistical Analysis

Data were analyzed as a completely randomized design experiment with

uneven replication using the General Linear Model of SAS (Statistical Analysis

Systems Institute Inc., 1992); orthogonal contrasts: Control vs. CLA

supplementation prior to weaning and Control vs. SAFF supplementation after

weaning were used to compare differences in treatment means.

3.4. Results

3.4.1. Growth Performance

The average daily growth rate of lambs during pre-weaning was 305 ± 66 g

per day and difference due to treatment was not observed. The rate of gain of

lambs, post-weaning, in the control, CLA, SAFF treatment groups was 382, 301

and 349 ± 21 g per day and dry matter intake was 1.43, 1.24 and 1.21 ± 0.06 kg

per day, respectively. The rate of gain of lambs in the CLA treatment group was

less than that of the lambs in the control group while the dry matter intake of

lambs in both the CLA and SAFF treatment groups was lower than that of lambs

in the control group. The feed conversion efficiency tended to be better for lambs

in the SAFF treatment (3.5) relative to those in the CLA treatment (4.1) and neither treatment was different from the control

3.4.2. Total Lipid Content

Quantities of TL extracted from the various tissues are shown in Table 1. No significant differences in TL extracted from samples of the diaphragm, leg, rib and liver obtained from lambs on the three dietary treatments were observed. However, the quantity of lipid extracted from the subcutaneous fat of the lambs provided CLA prior to weaning was considerably lower ($P < 0.05$) than the control, whereas that from lambs fed the SAFF supplemented diet, post-weaning, was significantly higher, compared with that of lambs fed the unsupplemented diet. The liver of lambs fed CLA contained significantly lower ($P < 0.05$) amounts of lipids when compared with that of lambs in the control treatment.

3.4.3. CLA Concentration

Significant differences were observed when the CLA concentration in the various tissues was compared across dietary treatment (Table 2). Supplementation of lamb diets with SAFF resulted in increases ($P < 0.05$) in the amount of CLA in the tissues when compared with that of animals fed the unsupplemented control diet. The CLA content was not affected in any of the tissues examined from lambs fed CLA-supplemented milk replacer prior to weaning.

3.4.4. Fatty Acid Composition

The tissue compositions of other non-volatile major straight chain fatty

acids are presented for the diaphragm, leg and rib muscle, and adipose and liver tissue in Table 3. The C16:0 content was not significantly affected by CLA supplementation in any of the tissues examined. However, SAFF supplementation significantly increased C16:0 content in diaphragm muscle when compared with lambs fed the unsupplemented diet. The C18:0 content of adipose tissue was significantly reduced with CLA supplementation but other tissues were not affected. Dietary supplementation with CLA pre-weaning had no effect on C18:1 content in any of the tissues studied. However with SAFF supplementation, the C18:1 content was significantly reduced in all the tissues. The C18:2 content was significantly reduced with CLA supplementation in diaphragm muscle and adipose tissue, but not in leg and rib muscle and liver. Supplementation with SAFF increased ($P < 0.05$) C18:2 and decreased C18:3 content in all the tissues examined. The C18:3 content of muscle tissue was not affected by CLA supplementation, but there was a significant decrease in the level of this fatty acid in adipose tissue and a significant increase in the liver tissue.

3.5. Discussion

The growth rate of lambs in this study was within the range for lambs that have moderate to high growth potential as defined by NRC (1985). The absence of differences in feed conversion efficiency was due to lower intakes in lambs in the CLA and SAFF treatment groups relative to that observed for control lambs

Conjugated linoleic acid concentration of tissues is higher in ruminants

compared to nonruminants. In the first feeding approach, CLA was fed to preweaned lambs for direct deposition into the tissues. Secondly, the diet was supplemented with LA rich SAFF to enhance ruminal bacterial activity for the conversion of linoleic acid to CLA isomers.

Direct feeding of CLA to pre-weaned lambs did not increase the CLA content in any of the examined tissues. The CLA was probably metabolized for energy by the growing lambs. However there was a highly significant decrease in the amount of lipid extracted from the subcutaneous adipose tissue when compared with animals receiving the control diet. Similar highly significant reductions in body fat has been observed in pigs, mice, rats and chickens fed diets containing CLA (Dugan et al., 1997; Park et al., 1997; Pariza et al., 1996). The decrease in fat content in subcutaneous adipose tissue may be related to differences in induction of differentiation of fibroblasts to adipocytes. LA is considered to be a strong inducer for differentiation of fibroblasts to adipocytes via activation of peroxisome proliferation activator receptor -gamma (Lazar, 1998). The change in configuration of C18:2 may disable the system to recruit fibroblasts to differentiate into adipocytes and may be the reason for the lower fat content in mature adipose tissue in lambs fed CLA prior to weaning. However, in weaned lambs, supplemented with SAFF, the availability of CLA from rumen did not decrease fat content of adipose tissue, suggesting that CLA is not effective in restricting lipid accumulation once fibroblast differentiation into adipocytes has occurred.

The decrease in the liver lipid accumulation with dietary CLA in lambs was

opposite to the observation in rodent liver (Belury and Kempa-Steczko, 1997), which suggested that there may be a species difference. Nevertheless, these data support the suggestion by Belury and Kempa-Steczko (1997) that dietary CLA may have a role in lipid transport and metabolism.

A consistent significant increase in CLA content in all the tissues was observed with SAFF supplementation. These results indicated that provision of the precursor to CLA, LA, in the diets of ruminants is required to increase the CLA content of the body tissues. It is apparent that CLA produced *in situ* by isomerization of LA during biohydrogenation in the rumen is an effective method of increasing the CLA content of tissues. Similar increases in CLA content in milk have been observed in recent studies (Kelly et al., 1998; Mir et al., 2000). To our knowledge, effects of oil supplementation to the diet on CLA content in ruminant muscle, adipose and liver tissues have not been reported previously. The concentration of CLA in muscle samples from control lambs ranged from 0.64 to 3.13 mg CLA/g lipid and were within the range of values reported for various muscle tissue from lambs (Hansen and Czochanska, 1976; Fogerty et al., 1988). However, values for muscle from control lambs in the present study were lower than the value of 5.6 mg CLA/g lipid in lambs (Chin et al., 1992). In all these studies meat from lambs was purchased from retail stores and thus it was not possible to determine the effect of feeding regimen on CLA content. In the present study, SAFF oil supplementation increased the CLA content in rib muscle to 8.4 mg CLA/g lipid, which was 1.7- fold higher than in the rib muscle from control animals. This value was greater than the CLA content (7.4 mg

CLA/g lipid) in beef round muscle from grass fed cattle (Shanta et al., 1997). In this study the average content of CLA for leg and rib muscle for lambs in control and SAFF treatment was 76.6 and 178.6 mg/100 g tissue, respectively. At a consumption rate of 250 g/d of lamb per day, the intake of CLA would be approximately 192 mg and 446 mg from meat from lambs from the two treatments respectively, and substantially greater than the 55 mg/d of CLA that resulted in reducing incidence of breast cancer in women (Knekt et al., 1996), but considerably less than the 3.5 g/d of CLA determined by extrapolation from rat studies, to promote of human health benefits. (Ha et al ., 1989)

Content of CLA in liver and adipose tissue from lambs fed SAFF oil were not as high as the values of 12.6 and 16.9 mg CLA/g lipid reported by Banni et al.. (1996) in liver and adipose, respectively. This was perhaps due to the   : differences in the age of the animals and dietary conditions. In the present study, the average age was about 3.5 months and the lambs received milk replacer and high concentrate diets, while the suckling lambs in the study by Banni et al (1996) were one month of age and were nursed by ewes grazing on grass pasture.

Although the primary objective of this investigation was to determine the effect of dietary supplementation with CLA (pre-weaning) or SAFF (post-weaning) on CLA content of tissues, the composition of other non-volatile fatty acids such as C16:0 and C18 straight chain fatty acids were also affected by dietary treatments. The effect of CLA supplementation on fatty acid composition in lamb tissue has not been previously reported, but present data indicate that

there was substantial variation among tissues. In rats, C18:0 concentration in liver has been reduced with a CLA-supplemented diet (Belury and Kempa-Steczko, 1997),whereas in the present study, the C18:3 concentration in lamb liver was significantly increased. The increase in C18:2 content of all the tissues with SAFF supplementation of the diet was consistent with observations in other studies cited by Rule et al., (1995), that have utilized vegetable oil in lamb diets. The decreases in C18:1 and C18:3 content in lamb tissues in the present study, however, have not been reported previously. From a human health perspective, an increase in CLA content is beneficial (Ip, 1997) but a decrease in C18:1 in tissue is not desirable.

In conclusion, supplementation of lamb feedlot diet with a rich source of LA resulted in a significant increase in the CLA content of various tissues. Direct CLA supplementation of pre-ruminant lambs did not influence CLA content in the tissues. In this case, CLA may have modulated fatty acid metabolism as has been observed in non-ruminants.

Table 1

The effect of dietary supplementation with conjugated linoleic acid (CLA) or safflower oil (SAFF) on lipid concentration (%) in various tissues.

Tissue	Diet treatment			SEM	Contrasts: Control vs	
	Control	CLA	SAFF		CLA	SAFF
Muscle						
Diaphragm	26.9	31.5	25.0	1.88	NS	NS
Leg	12.8	13.9	14.8	1.18	NS	NS
Rib	49.8	51.0	41.1	3.57	NS	0.09
Adipose	71.7	58.7	82.6	3.00	*	*
Liver	13.6	11.1	13.5	0.77	*	NS

SEM, Standard error of the mean.

NS, Not significant.

*, significant P>0.05.

Table 2

The effect of dietary supplementation with conjugated linoleic acid (CLA) or safflower oil (SAFF) on CLA concentration in various tissues.

Tissue	Diet treatment			SEM	Contrasts: Control vs	
	Control	CLA	SAFF		CLA	SAFF
Muscle						
Diaphragm	0.84	0.83	2.60	.0174	NS	***
Leg	1.78	1.50	4.41	0.432	NS	***
Rib	3.13	3.40	8.41	0.473	NS	***
Adipose	2.77	2.60	7.33	0.232	NS	***
Liver	1.72	1.59	3.53	0.349	NS	***

SEM, Standard error of the mean.

NS, Not significant.

***, signifiant P>0.001

Table 3

The effect of dietary supplementation with conjugated linoleic acid (CLA) or safflower oil (SAFF) on fatty acid concentration (weight %) in various tissues.

Fatty acid	Diet treatment			SEM	Contrasts: Control vs	
	Control	CLA	SAFF		CLA	SAFF
Diaphragm						
16:0	22.7	24.9	25.1	0.49	NS	*
18:0	19.1	18.1	19.3	0.52	NS	NS
18:1	47.7	49.1	41.4	1.12	NS	***
18:2	9.4	6.6	13.4	0.65	***	***
18:3	1.0	1.1	0.4	0.09	NS	***
Leg						
16:0	27.5	29.3	29.7	0.45	NS	NS
18:0	14.7	12.5	16.9	0.47	NS	NS
18:1	47.9	48.2	39.7	0.62	NS	***
18:2	8.6	8.6	12.4	0.49	NS	***
18:3	1.1	1.3	1.7	0.06	NS	***
Rib						
16:0	30.0	32.0	32.1	0.49	NS	NS
18:0	17.4	14.9	20.0	0.66	NS	NS
18:1	45.5	45.9	38.0	0.75	NS	***
18:2	5.7	5.4	8.5	0.33	NS	***
18:3	1.3	1.5	0.7	0.12	NS	***
Adipose						
16:0	28.2	30.9	32.4	0.64	NS	NS
18:0	12.1	8.2	14.3	0.81	***	NS
18:1	52.5	56.9	43.2	1.35	NS	***
18:2	5.8	3.3	8.4	0.33	***	***
18:3	1.2	0.5	0.7	0.10	***	***
Liver						
16:0	23.4	23.4	21.6	0.55	NS	*
18:0	33.3	34.6	32.8	0.74	NS	NS
18:1	25.0	22.2	21.3	1.26	NS	*
18:2	16.2	17.2	22.7	0.98	NS	***
18:3	1.9	2.5	0.8	0.17	*	***

SEM, Standard error of the mean.

NS, Not significant.

*, significant P>0.05.

***, significant P>0.001

4. Incorporation of Conjugated Linoleic Acid (CLA) into Glycerolipids of Lamb Tissues.

4.1. Abstract

The meat of ruminants contains conjugated linoleic acids (CLA) that have been shown to have nutraceutical value in preventing cancer and reducing the risk of heart disease. CLA are produced in the rumen by isomerization of linoleic acid. The objective of this study was to examine the level of CLA (composed mainly of the *cis*-9, *trans*-11-octadecadienoate isomer) in triacylglycerols (TAGs) of lamb tissue and the distribution of CLA on the TAG molecule as influenced by dietary regime. The CLA content of phospholipid (PL) from these tissues was also examined along with the positional distribution of CLA in PL of liver tissue. Thirteen lambs were randomized to three dietary treatments:

1) control diet (no supplement); 2) CLA supplementation (0.33 g d^{-1} for 21 d prior to weaning) to milk-replacer of pre-ruminant lambs; and 3) feeding linoleic acid-rich oil (Safflower oil, 6% DM- safflower) to weaned ruminating lambs. Lambs were slaughtered at an average weight of 45 kg and tissue samples were procured from diaphragm, rib and subcutaneous (SC) fat tissue for determination fatty acid (FA) composition of TAG and PL, and positional distribution of FAs on TAG. Liver tissue was procured for determination of the FA composition of PL and positional distribution of FAs on PL. Safflower oil supplementation in the diet resulted in an increase in CLA content of the TAG from diaphragm, rib and

SC fat by about 2.5- to 3-fold ($P < 0.05$). CLA was localized to the *sn*-1/3

positions of TAG suggesting that acyltransferase in these tissues had a lower

prference for CLA moieties compared to other fatty acyl moieties. Animals that

received pre-formed CLA, however, had increased proportions of CLA at the *sn*-2

position of TAG from SC fat. Safflower supplementation in the diet had no effect

on the CLA content of PL from diaphragm, rib and SC fat tissue suggesting that

CLA was preferentially incorporated into the TAG of these tissues. Positional

distribution studies with PL from liver tissue, however, indicated that CLA was

exclusively localized to the *sn*-2 position of the glycerol backbone.

4.2. Introduction

Conjugated linoleic acids (CLA) are produced in ruminant animals by the

bacterium *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1966). CLA isomers are

intermediary metabolites in the bioconversion of dietary linoleic acid (18:2) to

stearic acid (18:0) by *Butyrivibrio fibrisolvens*. Currently, CLA are defined as a

combination of several positional and geometric isomers of 18:2 with double

bonds predominantly at the 9 and 11, 10 and 12, or 11 and 13 carbon atoms,

with various combinations of *cis* and *trans* configuration at each double bond.

Although CLA represent a relatively minor component of the total FA composition

of foods, CLA exhibit chemoprotective properties against carcinogens when

consumed at very low levels (<1% of the diet or 3.5 g d^{-1}) (Ip, 1997; Ha *et al.*,

1989). Knekt *et al.* (1996) reported reductions in incidence of breast cancer in

women consuming 55mg CLA d^{-1} from milk.

In general, meat and dairy products from ruminants have been shown to

contain more CLA than products from nonruminants (Fogerty *et al.*, 1988; Jiang

et al., 1996). The concentration of CLA in beef has been shown to range from

1.7 to 8.5 mg CLA g^{-1} fat and is dependent on the nature of the feed (Shantha *et*

al., 1994; Mir *et al.*, 1999). Canola oil or full-fat rapeseed supplementation of

diets fed to goats or dairy cows, respectively, increased CLA content in milk (Mir

et al., 2000; Stanton *et al.*, 1997). In a study using rats, a diet that included 5%

free 18:2 significantly increased the CLA concentration of both the neutral lipid

(NL) and phospholipid (PL) fractions of liver tissue samples (Chin *et al.*, 1994a).

As well, inclusion of pre-formed CLA in rat diets resulted in a 6-fold increased in

the CLA content of the PL fraction compared to the controls (Ip *et al.*, 1991). In

a more recent study using mice, a dose dependent increase in CLA concent was

observed in epidermal NL and PL as CLA in the diet was increased from 0% to

1.5% (Kavanough *et al.*, 1999). CLA and conjugated diene intermediates have

also been found in the PL fraction from lamb liver (Banni *et al.*, 1996).

In section 3 of this thesis, it was demonstrated that safflower

supplementation in the diet enhanced the CLA content of various lamb tissues

through provision of the18:2 precursor of CLA (Mir *et al.*, 2000). In the current

study, lipid samples were isolated from rib, diaphragm, SC fat and liver of lambs

fed either CLA as a free acid pre-weaning (pre-ruminating), or a high 18:2 diet

post-weaning (ruminating) to investigate effects on the CLA content of TAG and

PL. Analysis of the positional distribution of CLA in TAG from diaphragm, rib and

SC fat was conducted in order to gain some insight into the mechanism of CLA

incorporation into glycerolipids. PL from liver tissue was included in the

positional distribution studies for comparative purposes.

4.3. Materials and Methods

4.3.1. Lambs and Diets

Thirteen four week old crossbred (Suffolk x Dorset) lambs (avg. wt. 12.5 ±

3.43 kg) were removed from ewes and placed in two pens with free access to

milk-replacer from an artificial ewe feeding apparatus for 21 d. The lambs had

free access to a pelleted creep feed containing barley grain, alfalfa and soybean

meal. Each of the lambs in the control treatment (5 lambs) and those designated

to receive the 6% safflower oil diet (4 lambs) upon weaning were given 50 mL of

milk replacer containing 5 mL olive oil (carrier), daily by bottle. Lambs in the CLA

treatment group (4 lambs) received 50 mL milk-replacer containing 0.33 g CLA

(Pharmanutrients, Lake Bluff, IL, USA) dissolved in 5 mL of olive oil daily. After

21d, the lambs (avg. wt. 18.9 ± 3.38 kg) were weaned off the artificial ewe, and

housed in individual pens and provided with a pelleted diet ad libitum. The lambs

on the control and those that received CLA supplemented milk-replacer (pre-

weaning) were supplied with the pelleted diet, while the lambs on safflower oil

received the pelleted diet supplemented with 6% safflower oil on a dry matter

basis. Safflower oil purchased from a commercial source was guaranteed to

contain 78% 18:2. Chin *et al.* (1992) has reported that commercial safflower oil

contains 0.7 mg CLA g^{-1} lipid. The ingredient composition of the pelleted diet

was: barley, 56.2%; dehydrated alfalfa, 29.5%; soybean meal, 9.0%; molasses,

2.0%; sheep mineral, 1.05%; maxi-pel, 1.0%; $CaPO_4$, 0.5%; NH_4Cl, 0.5%;

dicalcium phosphate, 0.2%; vitamin A (10 million IU kg^{-1}), vitamin D (1 million IU kg^{-1}), and vitamin E (100 000 IU kg^{-1}), 0.0333%; and deccox premix, 0.0181%. The crude protein of this diet was 16.6%. Lambs were fed for 80±1.8 d on this diet. The average weight at slaughter was 45.9 ± 2.9 kg.

4.3.2. Tissue Samples

At the time of slaughter, samples were procured from diaphragm, SC fat and liver. A double chop from the 12th and 13th ribs was sampled from each animal at the time of cutting. All tissue samples were obtained from the same half of the carcass. Tissue samples were stored in Whirl-Pak bags with as little air as possible, kept on ice for approximately 30 minutes and then frozen in liquid N$_2$. Tissues were stored at -20°C until use. Samples were thawed at 4°C and subsamples were taken and ground with a hand grinder until completely blended, and then refrozen and stored at -20°C.

4.3.3. Lipid Extraction

Lipids were extracted using CHCl$_3$:MeOH (2:1, v/v) (Folch et al., 1957). Stored tissue was thawed at 4°C and 500 mg of tissue were weighed into a test tube and homogenized in 5 mL of CHCl$_3$:MeOH (2:1, v/v), using a Kinematica homogenizer model PT-10-35 (Brinkman Inst., Rexdale, ON, Canada) at speed setting 6 for 30 seconds. The probe was rinsed with 5 mL CHCl$_3$:MeOH (2:1, v/v) and the rinse and homogenate were combined and centrifuged at 3000 rpm (1250 x g) for 30 min. The supernatant was washed two times with 2 mL 0.29% NaCl (w/v), and the final organic phase was passed through a small column of

anhydrous Na_2SO_4 to remove water from the sample. The lipid extract was then evaporated to dryness under N_2 gas with gentle heating (37°C to 40°C). The total lipid (TL) was dissolved in $CHCl_3$ to a final concentration of 150 mg ml^{-1} and stored at -20°C under gaseous N_2.

4.3.4. Lipid Class Separation

Using the method of Juaneda and Rocquelin (1985) modified by Chin et al (1994a), 30 - 60 mg of TL in 500 µL - 1 mL $CHCl_3$ was fractionated into NL and PL fractions. The TL was loaded onto a silica cartridge. (Sep-Pak Classic, Waters Limited, Mississauga, ON) and the fraction eluted in three steps. The NL fraction was eluted with 20 mL $CHCl_3$ injected by syringe at a flow rate of approximately 25mL min^{-1}. Monoacylglycerol (MAG) was eluted with 10 mL $CHCl_3$:MeOH (98:2, v/v) at a flow rate of approximately 20 mL min^{-1}. The PL fraction was eluted with 30 mL methanol at a flow rate of 25 mL min^{-1} The PL and NL fractions were evaporated to dryness under N_2 and dissolved in $CHCl_3$ to a final concentration 10mg mL^{-1} then stored under N_2 gas at -20°C.

4.3.5. TAG Isolation

Two hundred microliters of 150 mg TL ml^{-1} (30 mg TL total) were applied to a pre-run EM Science HPTLC- Fertigplatten Kieselgel 60 silica gel plate (10 x 20 cm x 0.25 mm thick) (VWR, Canlab, Mississauga, ON). Fifty microliters of 10 mg triolein mL^{-1} were applied as a reference standard (Sigma Aldrich Canada, Oakville, ON, Canada). Lipids were separated by one ascension of hexane:ether:acetic acid (80:20:1 v/v/v) as a solvent system (Pomeroy et al.,

1991). The isolated TAG was dissolved in $CHCl_3$ to a concentration of 10mg mL^{-1} and stored under N_2 gas at -20°C.

4.3.6. Digestion of TAG Using Pancreatic Lipase

Using the method of Myher and Kuksis (1979), pancreatic lipase was used to generate sn-2 monoacylglycerol (2-MAG) from TAG. Two milligrams TAG were evaporated to dryness under N_2 gas. The dried TAG was suspended in 500 μL of pancreatic lipase buffer (1M Tris-HCl, pH 8.0, 10% (w/v) Gum Arabic, 0.23 MCaCl$_2$ (w/v)) by sonication. Five hundred microliters of pancreatic lipase buffer containing 8 mg mL^{-1} pancreatic lipase were added to the TAG suspension, the mixture was vortexed briefly and incubated at 37°C for 1h in a shaking water bath. The reaction was stopped with 500 μL of 0.1N acetic acid and the lipid was extracted three times with 2 mL diethyl ether. Ether extract was passed over anhydrous Na_2SO_4, combined and then evaporated to dryness under N_2 gas. The extracted lipid was redissolved in 100-200 μL $CHCl_3$ and applied to boric acid TLC plates. Fifty microliters of a 10 mg mL^{-1} solution of monolein (Sigma Aldrich Canada, Oakville, ON, Canada) were applied as a reference standard. Lipids were separated by one ascension of $CHCl_3$:acetone (88:12, v/v) as a solvent system (Myher et al, 1996).

4.3.7. Digestion of Liver PL Using Phospholipase A$_2$

Two milligrams of liver PL were sonicated for ten minutes in 1 mL of borate buffer (diethyl ether: borate pH 8.9 (1:1 v/v)). Twenty five units of

phospholipase A₂ from *Apis mellifera* venom (Sigma Aldrich Canada, Oakville, ON, Canada) were added and the mixture incubated at 37°C for 2h with gentle shaking. The reaction was stopped with 500 µL of 0.1N acetic acid and the ether evaporated under N₂ gas. The lipid was extracted from the reaction three times with 2 mL of CHCl₃:MeOH (2:1 v/v). The extracts were passed over anhydrous Na₂SO₄ and combined. After evaporating to dryness under N₂, the lipid was resuspended in 100 - 200 µl CHCl₃ and applied to boric acid TLC plates. Fifty microliters of 10 mg mL⁻¹ solution of lysophosphatidic acid (LPA) (Sigma Aldrich Canada, Oakville, Ont., Canada) was applied as reference standard. Lipids were separated by one ascension of CHCl₃/EtOH/H₂O/triethylamine (35:30:6:35, v/v) as a solvent system.

4.3.8. Elution of TAG and MAG from Silica Gel

TAG and MAG standards were visualized with iodine vapor. The silica containing the fraction of interest was removed from the TLC plate with a glass microscope slide onto glasine paper and transferred to a methanol washed test tube. Lipids were eluted from silica by extracting twice with 5 mL of CHCl₃ and once with 2 mL of the solvent. The slurry was shaken vigorously and centrifuged at 400 x g for 5 minutes. Each eluted fraction was passed over anhydrous Na₂SO₄. The dehydrated fractions were combined and was stored at -20°C under N₂ gas.

4.3.9. Elution of LPA from Silica Gel

The LPA standard was localized by spraying the plate with a solution of 5

mg primulin mL^{-1} in acetone:water (4:1, v/v) and visualizing under ultraviolet light. The silica containing the fraction of interest was removed from the TLC plate with a glass scraper onto glasine paper and transferred to a test tube. LPA was eluted from the silica by extracting sequentially with 4 mL portions of developing solvent and $CHCl_3:MeOH:H_2O$ (5:5:1, v/v). The slurry was shaken vigorously and centrifuged at 400 x g for 5 min. The extracts were combined and partitioned with 4mL H_2O. The organic phase was passed over anhydrous Na_2SO_4 and stored at -20°C under gaseous N_2.

4.3.10. Preparation of Boric Acid TLC Plates

Boric acid TLC plates were prepared using a slurry of 60 g Silica gel G (VWR Canlab, Mississauga, ON, Canada) and 160 mL 0.4 M boric acid to make five 20 cm x 20 cm x 0.5 mm thick TLC plates. The plates were allowed to air dry then were heated for 2h at 120°C. They were then stored in the presence of desiccant until use.

4.3.11. Analysis of FA Composition

Tetramethylguanidine was used to transmethylate the lipid samples (Shantha et al., 1993; Schuchardt and Lopes, 1988). The lipid samples were dried under gaseous N_2 with 2 µL of 20mg C21:0 methyl ester mL^{-1} added as an internal standard. The dried lipid was suspended in 500 µL MeOH: Tetramethylguanidine (4:1, v/v) and incubated in boiling water bath for 30min. Five milliliters of saturated NaCl solution were added after cooling to room temperature, and the lipids were extracted three times with 2 mL of petroleum

ether. The extracts were combined and passed over anhydrous Na_2SO_4. The lipid extract was evaporated to dryness under N_2 and redissolved in 100 μL hexane. FA analysis was carried out as described by Mir *et al.*, (1999) on a Supelcowax-10, 30 m x 0.25 mm x 0,25μm column (Sigma Aldrich Canada, Oakville, ON, Canada) installed in an HP5890 Series Plus II gas chromatograph equipped with a flame ionization detector and splitless injection. The initial temperature was 50°C. This was increased to 200°C at a rate of 25°C per minute. Following this the temperature was then increased, sequentially, from 200°C to 220°C and 220°C to 240°C at a rate of 1°C per min and 15°C per min, respectively. Identification of FAs was by comparison to retention times of known standards (Sigma Aldrich Canada, Oakville, ON, Canada) and the peaks were quatified based on the area under each peak. Percentages of FAs were calculated from the total of the peaks in the chromatogram. Gas liquid chromatography peaks for all CLA isomers were pooled and reported as a percentage of the total FA composition.

4.3.12. Calculations and Statistical Analysis

The FA composition of the *sn*-1/3 positions of the TAG was calculated from the mean values obtained from the TAG (combined *sn*-1, *sn*-2 and *sn*-3), and 2- MAG (*sn*-2) FA analysis as follows.

sn-1/3% = (TAG% x 3 - 2-MAG%) / 2   (Kagawa *et al.*, 1996)

The FA composition of the liver PL *sn*-2 position was calculated from the mean values obtained from the PL (combined *sn*-1 and *sn*-2) and LPA (*sn*-1) analysis.

sn-2% = PL% x 2 – LPA%

FA composition data was analyzed using the JMP IN statistical package (Statistical Analysis Systems Institute Inc., 1989 - 1997) using a Dunnett's test to compare the results of control diets versus diets supplemented with pre-formed CLA and results of control diets versus diets supplemented with safflower oil.

4.4. Results

4.4.1. FA Composition of TAG from Diaphragm, Rib and SC Fat

The FA compositions of the TAG fractions of diaphragm, rib and SC fat are shown in Tables 1, and 3. In general, regardless of diet, oleic acid (18:1) was the major component of the TAG (47.6% - 57.4%) in these three tissues. This was followed by palmitic acid (16:0) (23.0% - 30.9%), stearic acid (18:0) (8.8% -18.8%), 18:2 (4.4% - 6.1%), CLA (0.2% - 1.5%) and, ∝-linolenic acid (18:3) (0.5% - 1.2%). The cis 9, trans 11 isomer was the predominant form of CLA in all cases. The proportion of CLA present in the TAG of the safflower fed animals was significantly higher (P < 0.05) than in the control in all three tissues. No significant change in the CLA content of TAG was observed in the tissues of the CLA supplemented group in comparison to control. The CLA content of the TAG fraction of the diaphragm increased by more than 2.5-fold in animals receiving the safflower oil dietary treatment (Table 1), increasing from 0.3% in the control group to 0.8% in safflower oil fed group. The CLA content of the TAG fraction of the rib increased by the same amount in animals receiving the safflower oil dietary treatment (Table 2). There was a significant (P < 0.05)

decrease in the percentage of 16:0 and ∝-18:3 present in the TAG from the rib

tissue of safflower oil fed animals (Table 2). The proportion of 16:0 in the rib

TAG decreased from 29.4% in the control group to 25.7% in safflower oil fed

animals whereas 18:3 decreased from 1.0% of the TAG in the control to 0.5% of

the TAG FAs in the safflower oil fed group. The decrease in content of these two

FAs was not observed in the TAGs of the diaphragm and SC fat indicating that

the effect of safflower oil supplementation on FA composition of TAG may be

tissue or fat depot specific. The CLA content of the TAG of the SC fat increased

in the TAG from the safflower oil fed group, from 0.5% in the control to 1.5% in

the safflower oil fed animals (Table 3). In SC fat TAG, the percentage of 18:0

was lower than in other tissues, ranging from 8.8% - 10.7%, versus 17.1% -

18.8% observed in diaphragm TAG (Table1) and 25.7% - 30.9% observed in rib

TAG (Table 2). The percentage of 18:1 in subcutaneous fat TAG (Table 3) was

slightly higher than in other tissues ranging from 55.1% - 57.4%, versus 48.7 % -

52.3% in diaphragm TAG (Table 1) and 47.6% - 49.1% in rib TAG (Table 2).

4.4.2. Positional Distribution of FAs in TAG from Diaphragm, Rib and SC Fat

The FA composition of the sn-2 and sn-1/3 positions of diaphragm, rib and

SC fat TAG as determined by pancreatic lipase digestion are shown in Tables 1-

3. No significant differences in the proportions of FAs in the sn-2 position of TAG

from the diaphragm, rib or SC fat were seen as a result of pancreatic lipase

digestion of TAG. Digestion of TAG with pancreatic lipase determined that CLA

was more abundant in sn-1/3 positions of TAG than in the sn-2 position. The

proportion of CLA detected at the sn-2 position was 0.2% or lower in all

categories with the exception of SC fat where the proportion of CLA in the *sn-2* position was 2.3% (Table 3). The proportion of CLA present in the *sn-1/3* positions of TAG from the three tissues of lambs that were fed with diet supplemented with safflower oil was significantly higher ($P < 0.05$) than in *sn-1/3* positions of TAG from controls. The FA composition at the *sn-2* position of TAG differed between the lipid extracted from the two muscle tissues (diaphragm and rib) and the lipid extracted from SC fat.

In the *sn-2* position of the TAG from the diaphragm, the most abundant FA was 18:1 comprising 40.2% - 53.6% of the FAs at that position (Table 1). Oleic acid was also the most abundant FA in the *sn-1/3* position comprising 51.4% - 58.0% of the combined *sn-1* and *sn-3* FAs. The percentages of 18:2 detected in the *sn-2* position ranged from 6.6% - 7.8% whereas the combined percentage for the *sn-1/3* positions range from 2.7% to 4.7%. Almost no CLA was observed in the *sn-2* position of the diaphragm TAG, with the exception of the control where CLA was detected at 0.15%. The proportion of CLA in the *sn-1/3* positions of TAG increased 4-fold from 0.3% in control animals to 1.2% the safflower oil fed group.

In the TAG from the rib, 18:1 was the most abundant FA in the *sn-2* position (43.2% - 45.0%) (Table 2). It was also the most abundant FA in the combined *sn-1* and *sn-3* positions (49.1% - 52.5%). The percentage of 18:2 detected in the *sn-2* position ranged from 4.3% to 7.0 %. The value for the combined *sn-1* and *sn-3* positions was 3.6% - 6.4%. CLA was detected in the *sn-2* position of TAG from rib in the animals on the control and safflower oil

supplemented diets at a level of 0.2%. In the sn-1/3 positions of TAG, the CLA content was significantly increased (P < 0.05) in the safflower oil supplemented animals (from 0.3% for rib controls to 1.1% in rib from animals that received safflower supplemented diets).

The most abundant FA in the sn-2 position of TAG from SC fat tissue was 18:1, which ranged from 60.4% in the safflower oil supplemented animals to 71.3% in the CLA supplemented animals (Table 3). CLA was detected in the sn-2 position of the SC fat tissue TAG from the CLA supplemented animals at 2.3% of the total FA at that position. The combined sn-1/3 CLA content was 2.2%, which was significantly higher (P < 0.05) than the 0.7% detected in the TAG from the control group.

4.4.3. FA Composition of PL from Diaphragm, Rib, SC Fat and Liver

The FA composition of PL was different for all the tissues studied but no dietary effect was observed. CLA was present in the PL of diaphragm, rib, SC fat and liver, but there was no significant change in the distribution of this FA within each tissue due to diet (Tables 4 and 5). The major FA of diaphragm PL was 18:2 (32.9% - 43.6%). The CLA content in the diaphragm was lower than in any of the other tissue PLs ranging from 0% - 0.03% (Table 4). The major FAs of rib PL were 18:1 (25.8% - 35.2%) and 18:2 (24.5% - 44.6%). The CLA content of rib PL ranged from 0.05% - 1.8% (Table 4). The major FA of SC fat PL was 18:1 (48.8% - 55.5%). The CLA content of SC fat PL ranged from 1.0% - 1.1%. The percentage of 18:2 present was the lowest of all tissues studied at 6.2% - 10.9% (Table 4). The most abundant FAs of liver PL were 18:1 (25.6% - 27%) and 18:0

(30.8% - 33.4%). The CLA content of liver PL ranged from 0.05% - 2.9% (Table 5).

4.4.4. Positional Distribution of FAs in Liver PL

Phospholipase A_2 digestion of liver PL determined CLA was present in the sn-2 position of the liver PL (Table 5). The sn-1 position of liver PL had a higher proportion of 16:0 (28.2% - 31.4%) than the sn-2 position (7.3% - 13.2%). The sn-2 position of liver PL had a higher proportion of 18:1 (36.3% - 38.2%) than the sn-1 position (13.0% - 23.6%) of liver PL.

4.5. Discussion

Basal diets supplemented by unprotected polyunsaturated fats in vegetable oils have been shown to have little effect on FA composition of adipose tissue and skeletal muscle sampled from various sites in steers (Christie, 1979). For example, Christie's work showed that supplementation with 6% safflower oil only resulted in a slight increase in the proportion of 16:0 and 18:0. Because dietary lipids are extensively hydrogenated in the rumen to produce saturated FAs, incorporation of higher levels of polyunsaturated fats in ruminant tissues is usually accomplished by the feeding of protected feed supplements that pass through the rumen and are absorbed in the small intestine unchanged. The 18:2 present in safflower oil is biohydrogenated in the rumen to ultimately produce 18:0. Intermediates of the biohydrogenation of 18:2 are CLAs (Kepler et al., 1966). In the current study, lambs fed a diet supplemented by 6% safflower oil showed a significant increase ($P < 0.05$) in the proportion of CLA in the TAG

fraction of diaphragm, SC fat and rib but no significant change in the content of other major FA components was observed. In section 3 of this thesis, increases of 2- to 4-fold in the CLA content of TL of tissues were reported for lambs fed a diet supplemented with 6% safflower oil (Mir *et al.*, 2000).

The polyunsaturated fat content in tissues of young ruminants has been shown to increase following feeding with milk replacers supplemented with 12.5 - 70 g d $^{-1}$ of sunflower oil for up to sixteen weeks (Christie, 1979). The analysis of the muscle and adipose tissue fats by Christie from animals on this supplemented diet contained up to 40% of 18:2 in the TAG fraction after 30 d. This increase in 18:2 content was accomplished due to the bypass of the rumen when the esophageal groove was closed while suckling. In this way, the components of the diet were absorbed in the small intestines as in non-ruminants. In the current study, the direct feeding of CLA to pre-weaned lambs for 21 d prior to weaning did not increase the CLA content of the TAG fraction of any of the tissues studied. The CLA may have been metabolized for energy by the growing lambs. The increased CLA content observed in the TAG of the animals receiving the safflower diet resulted in incorporation and storage of CLA in the TAG fraction of diaphragm, SC fat and rib of lambs. The higher proportion of CLA in the TAG of the SC fat, which was 2-fold that of diaphragm or rib, suggested that CLA was preferentially stored in SC fat.

In mammalian fat depots, unsaturated FAs have a tendency to be localized to the *sn*-2 position of TAG whereas saturated FAs tend to be localized to the *sn*-1 and *sn*-3 positions (Brokerhoff *et al.*, 1966). Changes in FA positional

distribution as affected by diet have been shown in bovine and ovine adipose tissues (Hawke *et al.*, 1977; Mills *et al.*, 1975; Smith *et al.*, 1998) and ovine liver (Mills *et al.*, 1975), but the mechanism responsible for these dietary induced changes is unknown. In previous studies, high proportions of additional 18:2, due to protected supplement feeding, incorporated into adipose tissues of ruminants were preferentially esterified to the *sn*-2 position and to a lesser extent the *sn*-3 position (Christie, 1979; Mills *et al.*, 1975). In the current study, the diaphragm, rib and SC fat TAG positional distribution analysis showed an increased proportion of CLA in the *sn*-1/3 position in the safflower diet group suggesting that lysophosphatidate acyltransferase in these tissues had a decreased preference for CLA moieties compared to other fatty acyl moieties. Lysophosphatidate acyltransferase (EC 2.3.1.51) catalyzes the acyl-CoA dependent acylation of LPA to generate phosphatidate (Lehner and Kuksis, 1996). In contrast, CLA was detected in the *sn*-2 position of TAG of SC fat from the CLA supplemented animals. This observation indicated that the feeding of CLA pre-weaning might have had an effect on the placement of the CLA on TAG from SC fat.

Previous studies on incorporation of CLA have shown that CLA is readily incorporated into the PL fraction of the mammary gland and forestomach tissues of rodents (Ha *et al.*, 1990; Ip *et al.*, 1991). There is also evidence that CLA is preferentially incorporated into the NL fraction (Belury *et al.*, 1996). In the current study, safflower supplementation in the diet had no effect on the CLA content of PL from diaphragm, rib and SC fat tissue suggesting that CLA was preferentially

incorporated into the TAG of these tissues. The lack of change in the FA composition of the PL fraction as a function of dietary manipulation indicated that there may be a threshold of CLA incorporation into the PL of ruminants. Belury *et al.* suggested that the biological activity of CLA might be more related to deposition of fat rather than through effects on PL alteration. The low levels of ⅀-18:3 in the PL of rib, liver and SC fat may be due to the competitive metabolism of CLA versus 18:2 to produce a conjugated form of 18:3 (Banni *et al.*, 1996), which was not quantified in this study. Banni *et al.* detected the intermediates of CLA elongation and desaturation incorporated into liver PLs along with the end product, conjugated arachidonic acid.

Tissue PLs have a characteristic positional distribution of FAs as in tissue TAGs. In general, saturated and unsaturated FAs of PL are located at the *sn*-1 and *sn*-2 position, respectively (Mills *et al.*, 1975). The results from the current study conformed to this trend.

In conclusion, supplementation of lamb feedlot diet with safflower oil as a source of unprotected 18:2 resulted in a significant increase in the CLA content of TAG of various tissues. Dietary manipulation had no significant effect on the CLA content of the PL suggesting that the FA isomers were preferentially incorporated into TAG. The highest proportions of CLA were observed in SC fat indicating that CLA was preferentially stored in this fat depot. CLA was not detected in any tissues examined in significant quantities at the *sn*-2 position of the TAG molecule. There was, however, an increased amount CLA in the *sn*-2 position of the SC fat from animals supplemented with pre-formed CLA.

Although CLA feeding pre-weaning had no effect on the total CLA content of the TAG, it influenced the positional distribution of CLA on the TAG molecule. CLA was exclusively localized to the *sn*-2 position of PL from liver under all dietary regimes. Studies on the specificity and selectivity for CLA of the acyltransferases involved in TAG bioassembly should provide further insight into the mechanism of CLA incorporation into glycerolipids.

Table 1

Effect of diet on FA composition of TAG and positional distribution of FAs in TAG from diaphragm.

Fraction	Diet	Fatty acid (wt.%)						
		16:0	18:0	18:1	18:2	18:3	CLA	n
TAG								
	CLA	24.9	17.1	52.1	4.4	1.0	0.4	3
	Control	23.0	18.8	52.3	4.8	0.8	0.3	5
	Safflower	26.2	17.5	48.7	5.8	0.9	*0.8	4
	SEM	1.10	0.91	1.02	0.27	0.22	0.04	
sn-2								
	CLA	28.1	23.8	40.2	7.8	0.0	0.0	3
	Control	20.8	18.6	53.6	6.6	0.16	0.15	5
	Safflower	26.1	22.7	43.2	8.0	0.0	0.0	4
	SEM	1.89	1.77	2.96	1.72	0.07	0.06	
sn-1/3								
	CLA	23.4	13.9	58.0	2.7	1.5	0.5	3
	Control	24.1	18.8	51.6	3.9	1.1	0.3	5
	Safflower	26.2	14.9	51.4	4.7	1.4	*1.2	4
	SEM	1.86	1.74	2.01	0.98	0.32	0.07	

*$P < 0.05$ by Dunnett's test compared to control.

CLA, CLA in diet as free acid pre-weaning; safflower, 6% safflower oil in diet post-weaning; control, no dietary treatment; SEM, standard error of the mean.

Table 2

The effect of diet on FA composition of TAG and positional distribution of FAs in TAG from rib.

Fraction	Diet	Fatty acid (wt.%)						
		16:0	18:0	18:1	18:2	18:3	CLA	*n*
TAG								
	CLA	30.9	15.3	47.6	4.7	1.2	0.2	4
	Control	29.4	16.8	47.7	4.8	1.0	0.3	5
	Safflower	*25.7	18.2	49.1	5.7	*0.5	*0.8	4
	SEM	0.83	0.89	0.45	0.45	0.09	0.04	
sn-2								
	CLA	27.5	22.3	43.2	7.0	0.0	0.0	4
	Control	29.1	22.4	45.0	3.1	0.15	0.2	5
	Safflower	28.7	24.7	42.1	4.3	0.0	0.2	4
	SEM	2.04	2.89	2.93	1.59	0.06	0.14	
sn-1/3								
	CLA	32.7	11.8	49.9	3.6	1.7	0.3	4
	Control	29.6	14.1	49.1	5.6	1.3	0.3	5
	Safflower	24.2	15.0	52.5	6.4	*0.8	*1.1	4
	SEM	2.05	1.84	2.12	1.20	0.14	0.08	

*$P < 0.05$ by Dunnett's test compared to control.

CLA, CLA in diet as free acid pre-weaning; safflower, 6% safflower oil in diet post-weaning; control, no dietary treatment; SEM, standard error of the mean.

Table 3

The effect of diet on FA composition of TAG and positional distribution of FAs in TAG from SC fat.

Fraction	Diet	Fatty Acid (wt.%)						
		16:0	18:0	18:1	18:2	18:3	CLA	*n*
TAG								
	CLA	28.8	9.8	55.1	4.2	0.7	0.5	4
	Control	25.5	10.7	57.4	5.1	0.8	0.5	5
	Safflower	27.9	8.8	55.1	6.1	0.7	*1.5	4
	SEM	1.29	1.11	2.05	0.59	0.08	0.13	
sn-2								
	CLA	12.3	8.2	71.3	5.0	1.0	2.3	4
	Control	15.3	9.7	67.1	7.9	0.0	0.0	5
	Safflower	20.9	15.3	60.4	3.3	0.0	0.0	4
	SEM	2.14	1.67	3.10	1.24	0.30	0.70	
sn-1/3								
	CLA	37.0	10.6	48.4	3.8	0.6	•0.4	4
	Control	30.5	11.2	52.5	3.8	1.3	0.7	5
	Safflower	31.4	5.5	52.4	7.4	1.0	*2.2	4
	SEM	2.04	1.97	3.09	0.93	0.26	0.46	

*P< 0.05 by Dunnett's test compared to control;

•Negative value due to low abundance of FA.

CLA, CLA in diet as free acid pre-weaning; safflower, 6% safflower oil in diet post-weaning; control, no dietary treatment; SEM, standard error of the mean.

Table 4

The effect of diet on FA composition of PL from diaphragm, rib and SC fat.

Fraction	Diet	Fatty Acid (wt.%)						
		16:0	18:0	18:1	18:2	18:3	CLA	n
Diaphragm								
	CLA	18.2	24.2	22.9	32.9	1.8	0.01	3
	Control	16.3	25.7	21.2	35.1	1.6	0.03	5
	Safflower	12.8	26.6	16.2	43.6	0.7	0.00	4
	SEM	1.33	1.11	1.68	2.70	0.16	0.01	
Rib								
	CLA	23.8	14.0	35.2	24.5	2.5	0.05	4
	Control	20.5	15.3	34.0	26.6	2.3	1.2	5
	Safflower	14.2	12.8	25.8	44.6	0.9	1.8	4
	SEM	1.60	1.49	3.10	5.13	0.20	0.55	
SC fat								
	CLA	23.8	17.5	48.8	8.4	0.4	1.1	4
	Control	26.2	19.0	46.8	6.2	0.6	1.1	5
	Safflower	18.3	14.1	55.5	10.9	0.2	1.0	4
	SEM	2.87	2.04	4.91	2.37	0.19	0.51	

*$P < 0.05$ by Dunnett's test compared to control.

CLA, CLA in diet as free acid pre-weaning; safflower, 6% safflower oil in diet post-weaning; control, no dietary treatment; SEM, standard error of the mean.

Table 5

The effect of diet on FA composition of PL and positional distribution of FAs in PL from liver.

Tissue	Diet	Fatty Acid (wt.%)						
		16:0	18:0	18:1	18:2	18:3	CLA	n
PL								
	CLA	22.3	33.4	27.0	14.7	2.0	0.5	4
	Control	22.5	32.0	25.6	15.2	1.8	2.9	5
	Safflower	17.8	30.8	30.0	18.2	0.6	2.6	4
	SEM	1.44	1.74	0.76	1.95	0.23	1.36	
sn-1								
	CLA	31.4	33.1	16.8	16.9	1.8	0.0	4
	Control	29.8	29.3	13.0	26.4	1.5	0.0	5
	Safflower	28.2	24.7	23.6	23.2	0.3	0.0	4
	SEM	2.55	4.06	4.23	3.79	0.33	0.0	
sn-2								
	CLA	13.2	33.7	37.2	12.6	2.2	1.0	4
	Control	15.2	34.7	38.2	4.0	2.1	5.7	5
	Safflower	7.3	37.0	36.3	13.2	0.9	5.3	4
	SEM	4.1	3.8	4.7	3.6	0.4	2.7	

*$P < 0.05$ by Dunnett's test compared to control.

CLA, CLA in diet as free acid pre-weaning; safflower, 6% safflower oil in diet post-weaning; control, no dietary treatment; SEM, standard error of the mean.

5. Summary and Future Directions.

In this study, lambs were supplemented with CLA at a rate of 0.33g CLA day^{-1} for 21 days prior to weaning or with a rich source of linoleic acid (6% safflower oil) in their diet after weaning until a slaughter weight of 45kg was reached. Supplementation of lamb diets with a rich source of linoleic acid resulted in significant increases (P < 0.05) in the amount of CLA in the TL and TAG of tissues when compared with that of animals fed the unsupplemented control diet. The CLA content of the TL or TAG was not affected in any of the tissues examined from lambs fed CLA-supplemented milk replacer prior to weaning. Digestion of TAG with pancreatic lipase determined that CLA is more abundant in sn-1/3 positions of TAG than in the sn-2 position. Dietary manipulation had no significant effect on the CLA content of the PL fraction of the TL. Determination of the positional distribution of FA on the liver PL indicated that CLA is localized to the sn-2 position of liver PL.

Development of methods to increase CLA content in the carcass of ruminants will enhance the nutraceutical value of meat products. The described research on dietary manipulation of CLA in sheep is a step in this direction. The effect of diet in promoting CLA incorporation into meat should also be examined in beef cattle. The dietary effects of increased LA in the diet on rumen function, bacterial biohydrogenation processes and CLA incorporation should be examined to determine optimal dietary regimes. Further investigation into the positional distribution of CLA on the TAG should be examined in ruminants to

determine the distribution of CLA between the *sn*-1 and *sn*-3 positions. The positional distribution of CLA on PL of tissues other than the liver should be carried out to determine, for comparative purposes, if the same positional distribution pattern of FA does exist in other tissue. To extend the knowledge of positional distribution of CLA as a group of isomers it would be useful to determine the presence, quantities and locations of individual isomers of CLA on TAG and PL. It would also be useful to examine the specificity and selectivity for CLA moieities by acyltransferases involved in the synthesis of TAG and whether or not there are tissue specific differences in enzyme action. As well, it would be useful to study possible differences in specificity and selectivity of acyltransferases for CLA isomers. In addition, the possible modulatory effects of CLA and individual CLA isomers on acyltransferases could be investigated. A detailed understanding of the regulation of TAG biosynthesis in relation to diet in ruminants will provide useful information of the development of a value added meat product.

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