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Conjugated Linoleic Acid: Biosynthesis and Nutritional Significance

D.E. Bauman and A.L. Lock

Abstract

The term conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid with a conjugated double bond system; milk fat can contain over 20 different isomers of CLA. CLA isomers are produced as transient intermediates in the rumen biohydrogenation of unsaturated fatty acids consumed in the diet. However, *cis*-9, *trans*-11 CLA, known as rumenic acid (RA), is the predominant isomer (up to 90% of total) because it is produced mainly by endogenous synthesis from vaccenic acid (VA). VA is typically the major biohydrogenation intermediate produced in the rumen and it is converted to RA by Δ^9-desaturase in the mammary gland and other tissues.

Biomedical studies with animal models have shown that RA as well as VA have anticarcinogenic and antiatherogenic properties, with the effects of VA being related to its conversion to RA. The anticarcinogenic effects have been observed for a wide range of cancer types, but the most impressive results have been reported in relation to mammary cancer. Of special importance, RA and VA are potent anticarcinogens when supplied as natural food components in the form of VA/RA-enriched butter. The functional food considerations of CLA isomers in dairy products realistically relate only to RA as the major isomer, although this should include VA because in humans it serves for the endogenous synthesis of RA. The RA and VA content in milk fat are directly related and they can be markedly enhanced through the use of diet formulation and nutritional management of dairy cows.

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Trans-10, *cis*-12 CLA is another CLA isomer in milk fat which can affect lipid metabolism. It is generally present at low concentrations in milk fat (typically <0.2% of CLA); under some dietary conditions, a portion of the rumen biohydrogenation shifts to produce more of this isomer, although it is still only a minor portion of total CLA. These dietary conditions are associated with milk fat depression and as little as 2 g/d of *trans*-10, *cis*-12 leaving the rumen will reduce milk fat synthesis by 20%. Because of the potency and specificity of this CLA isomer, it is being developed as a dairy management tool to allow for a controlled reduction in milk fat output.

CLA isomers in milk fat and how they relate to both animal agriculture and human health are rapidly expanding fields. Milk and dairy products offer exciting opportunities in the area of functional foods, and the functional properties of VA and RA in milk further serve to illustrate the value of dairy products in the human diet.

3.1. Introduction

An adequate supply of good-quality food is essential for human health and well-being. Milk and meat products derived from ruminants represent important sources of nutrients in human diets, providing energy, high quality protein, and essential minerals and vitamins (National Research Council, 1988; Demment and Allen, 2003). Nutritional quality is increasingly an important consideration in food choices because of the growing consumer awareness of the link between diet and health. Many foods contain microcomponents that have beneficial effects beyond those associated with their traditional nutrient content, and these are often referred to as "functional food" components. One such component in foods derived from ruminants is conjugated linoleic acid (CLA).

CLA refers to a mixture of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid) with a conjugated double bond system. The structure of two CLA isomers is contrasted with linoleic and vaccenic acids in Figure 3.1. The presence of CLA isomers in ruminant fat is related to the biohydrogenation of polyunsaturated fatty acids (PUFAs) in the rumen. Ruminant fats are relatively more saturated than most plant oils and this is also a consequence of biohydrogenation of dietary PUFAs by rumen bacteria. Increases in saturated fatty acids are considered undesirable, but consumption of CLA has been shown to be associated with many health benefits, and food products derived from ruminants are the major dietary source of CLA for humans. The interest in health benefits of CLA has its genesis in the research by Pariza and associates who first demonstrated that

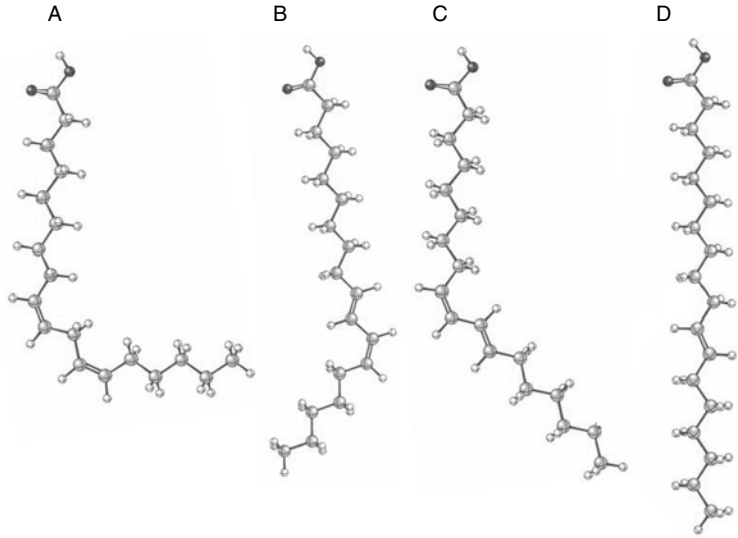

Figure 3.1. Chemical structures of linoleic acid (*cis*-9, *cis*-12 18:2; A), *trans*-10, *cis*-12 conjugated linoleic acid (B), rumenic acid (*cis*-9, *trans*-11 conjugated linoleic acid; C) and vaccenic acid (*trans*-11 18:1; D).

CLA isomers are functional food components when their search for mutagens in cooked meat instead identified CLA as an antimutagen (see Pariza, 1999). As a result of this discovery, research on CLA has increased exponentially over the last decade and a number of potential health benefits of CLA have been reported. The anticarcinogenic activity of CLA has been established clearly, but biomedical studies with animal models have identified an impressive range of additional positive health effects for CLA as summarized in Chapter 17. Particularly noteworthy is the fact that CLA is a potent anticarcinogen when supplied as a natural food component in the form of CLA-enriched butter as discussed later in this review.

The presence of CLA in ruminant milk has been known for more than 70 years and in this chapter we will first review the dietary sources of CLA and provide an overview of the analytical challenges associated with quantifying CLA in foods and biological samples. Secondly, we will review the origin of the different CLA isomers present in milk fat, developing the interrelationships between biohydrogenation intermediates produced in the rumen, synthesis of CLA in the tissues and the presence of these isomers in milk fat. Thirdly, we will highlight the nutritional and physiological factors

that effect the level of CLA in milk fat and discuss milk quality considerations for dairy products that have a naturally enhanced content of CLA. Finally, we will review the biological effects of CLA related to the dairy cow and dairy products. This will include its effects on milk fat synthesis in dairy cows, an area that has progressed rapidly and promises to contribute to our general understanding of the regulation of lipid metabolism. Our review of the biological effects will also include the significance of CLA in dairy products and its potential as a functional food component that benefits human health. Regular updates and references on the biology of CLA in dairy chemistry can be found at www.wisc.edu/fri/clarefs.htm and www.ansci.cornell.edu/bauman.

3.2. Dietary Sources

The predominant source of CLA in human diets is ruminant-derived food products. CLA is a fatty acid so it is present in milk fat and muscle fat. In the U.S., dairy products provide about 70% of the intake of CLA and beef products account for another 25% (Ritzenthaler *et al*., 2001). Similar values for the contribution of different food classes have been reported for other countries (see Parodi, 2003).

Scientists at the University of Reading, UK, first demonstrated that fatty acids obtained from summer butter differed from those obtained from winter butter by exhibiting a much stronger spectrophotometric absorption at 230 μm (Booth *et al*., 1933). It was subsequently concluded that the adsorption at this wavelength was due to a conjugated double bond pair (Moore, 1939). Parodi (1977) was the first to identify *cis*-9, *trans*-11 octadecadienoic acid as a fatty acid in milk fat that contained the conjugated double bond pair. As analytical techniques improved it was discovered that milk fat and body fat from ruminants contained many isomers of CLA that differ by position (e.g., 7–9, 8–10, 9–11, 10–12, 11–13) or geometric orientation (*cis-trans*, *trans-cis*, *cis-cis,* and *trans-trans*) of the double bond pair. The range of CLA isomers and their levels in milk and dairy products is summarized in Table 3.1. *Cis*-9, *trans*-11 is the major CLA isomer in ruminant fat, representing about 75 to 90% of the total CLA, and the common name of ''rumenic acid'' (RA) has been proposed for this isomer because of its unique relationship to ruminants (Kramer *et al*., 1998). The second most common isomer is *trans*-7, *cis*-9 CLA, representing about 10% of total CLA. Each of the other CLA isomers is at a low concentration when present, generally representing less than 0.5% of the total CLA in ruminant fat.

Table 3.1. Range of positional and geometric isomers of conjugated $C_{18:2}$ fatty acids in milk and dairy products. Adapted from Lock and Bauman (2004)*

Isomer	% of total CLA isomers
trans-7, *cis*-9	1.2–8.9
trans-7, *trans*-9	<0.1–2.4
trans-8, *cis*-10	<0.1–1.5
trans-8, *trans*-10	0.2–0.4
cis-9, *trans*-11	72.6–91.2
trans-9, *trans*-11	0.8–2.9
trans-10, *cis*-12	<0.1–1.5
trans-10, *trans*-12	0.3–1.3
cis-11, *trans*-13	0.2–4.7
trans-11, *cis*-13	0.1–8.0
trans-11, *trans*-13	0.3–4.2
cis-12, *trans*-14	<0.01–0.8
trans-12, *trans*-14	0.3–2.8
cis-cis isomers	0.1–4.8

* Data derived from seven studies where fatty acid analysis was carried out on milk samples (Precht and Molkentin, 1997; Piperova *et al*., 2002; Kraft *et al*., 2003; Shingfield *et al*., 2003; Kay *et al*., 2004), butter (Bauman *et al*., 2000) or cheese (Rickert *et al*., 1999).

3.3. Analytical Challenges

Biological samples generally contain multiple isomers of CLA, many at very low concentrations, and each may differ in their biological effects. Thus, the ability to determine the concentration of specific isomers is becoming increasingly important, and frequently a combination of analytical methods is required to quantify fully CLA isomers and related fatty acids (Christie, 2003; Kramer *et al*., 2004). The analysis of CLA typically requires their conversion to derivatives that can be separated from other fatty acids in the sample, usually by either gas chromatography (GC) or high-performance liquid chromatography (HPLC). Early investigations generally used high temperature, acid-catalyzed methylation to prepare fatty acid methyl esters, but subsequent work established that this procedure causes extensive isomerization, producing mainly *trans*/*trans* isomers. Therefore, data on the distribution of CLA isomers from early investigations are highly questionable (Yurawecz *et al*., 1999).

GC provides the basis for most analytical approaches reported in the literature, and the use of alkali-catalysed methylation has proven to be the most accurate method for analysis of CLA (Yurawecz *et al*., 1999). Sodium methoxide is the catalyst used most widely and has the advantage that it does not isomerize conjugated double bonds or form methoxy artifacts.

Kramer *et al.* (1997) demonstrated that this procedure completely methylated test samples containing mainly triglycerides, but it did not methylate free fatty acids. Since milk fat consists of ~98% triglycerides, the contribution made by other lipids can often be disregarded so that only a base-catalyzed methylation is necessary (Yurawecz *et al.*, 1999). The transmethylation procedure described by Christie (1982) with modifications by Chouinard *et al.* (1999a) to minimize the loss of highly-volatile short chain fatty acids, is used widely and recommended for the analysis of fatty acids in milk. In the GC analysis, the type of column is another important consideration. Long (100–120 m) highly polar columns are used typically and offer a reasonable degree of separation for CLA isomers, and especially *trans* 18:1 fatty acids (Christie, 2003). However, with typical GC procedures, *trans*-7, *cis*-9 CLA and RA co-elute, and other CLA isomers are often not separable, especially if there is a low concentration of one isomer relative to another.

Analysis of the CLA content and profile of animal tissues or biological fluids containing a mixture of lipid classes is more difficult. In order for all of the fatty acids to be methylated, a two-stage methylation procedure is recommended. Kramer *et al.* (1997) evaluated many different combinations of acid/base catalysts and concluded that the best compromise was the use of sodium methoxide followed by a mild acidic methylation, which resulted in the methylation of the majority of the fatty acids with minimal isomerization of the CLA isomers. However, mild boron triflouride or 1% methanolic sulphuric acid with a minimal temperature and reaction time are often used with good success.

Additional analytical methods are appropriate when a more complete characterization of the CLA isomers in biological samples is required. Most often, a combination of GC and silver ion-HPLC is used and permits excellent separation and identification of positional and geometrical isomers of CLA (see Adlof, 2003, and Kramer *et al.*, 2004, for detailed reviews of this approach). In addition, the use of gas chromatography-mass spectrometry (GC-MS) has become increasingly popular and represents a very powerful technique for identification of the position of double bonds in fatty acids (see Dobson, 2003), and the orientation of those bonds in CLA isomers (Michaud *et al.*, 2003).

In summary, the analysis of CLA can be simple or extensive. The particular objectives and the anticipated use of the analytical data will determine the extent to which individual CLA isomers need to be separated, identified and quantified (Christie, 2003). Methodology for the analysis of CLA and related fatty acids continues to evolve and it is recommended that the reader consult recent reviews and publications in this area before undertaking such analysis for the first time. We recommend Christie (2003) and Kramer *et al.* (2004) as excellent practical guides on the analysis of CLA.

In addition, periodic updates on CLA analysis can be found at www.lipidlibrary.co.uk.

3.4. Origin of CLA in Milk Fat

3.4.1. Lipid Metabolism in the Rumen

The diet of lactating dairy cows typically contains 4 to 5% fat, with the major PUFAs being linoleic and linolenic acids that are supplied mainly from dietary concentrates and forages, respectively. When dietary lipids enter the rumen, the initial step is hydrolysis of the ester linkages in the triglycerides, phospholipids, and glycolipids. Hydrolysis of dietary lipids in the rumen involves extracellular lipases that are produced by the rumen bacteria; there is little evidence to support significant roles for rumen protozoa and fungi, or salivary or plant lipases in rumen hydrolysis. The extent of hydrolysis of dietary lipids in the rumen is generally high (>85%), and a number of factors that affect the rate and extent of hydrolysis have been identified (see Harfoot, 1981; Doreau and Ferlay, 1994; Doreau *et al*., 1997b; Harfoot and Hazlewood, 1997 for reviews).

Biohydrogenation of PUFAs is the second major transformation that dietary lipids undergo in the rumen and this process first requires that the fatty acid is free. As a consequence, rates are always less than those of hydrolysis and factors that affect hydrolysis also affect rates of biohydrogenation. In the 1960s and 1970s, an extensive series of *in vitro* and *in vivo* studies examined rumen biohydrogenation (see Dawson and Kemp, 1970; Keeney, 1970; Harfoot, 1981; Harfoot and Hazlewood, 1997). Most biohydrogenation (>80%) occurs in association with the small dense (fine) food particles and this has been attributed to extracellular enzymes of bacteria either associated with the feed or free in suspension. A few species of rumen bacteria capable of carrying out the biohydrogenation reactions have been identified and predominant pathways have been elucidated. Kemp and Lander (1984) classified rumen bacteria involved in biohydrogenation into two groups based on their metabolic pathways. Group A included bacteria that could hydrogenate 18 carbon PUFAs to *trans* 18:1 fatty acids, whereas only a few species, characterized as Group B, could hydrogenate *trans* 18:1 fatty acids to stearic acid (Harfoot and Hazlewood, 1997). Thus, complete biohydrogenation of unsaturated fatty acids generally requires bacteria from both groups. However, recent research has identified an occasional exception where a specific bacterial strain can carry out the complete biohydrogenation of PUFAs to $C_{18:0}$ (see Palmquist *et al*., 2005).

The initial step in rumen biohydrogenation of linoleic and linolenic acids involves an isomerization of the *cis*-12 double bond to a *trans*-11

configuration, resulting in a conjugated dieonic or trienoic fatty acid (Figure 3.2). Next, is a reduction of the *cis*-9 double bond resulting in a *trans*-11 octadecenoic acid. Therefore, RA is an intermediate formed only during the biohydrogenation of linoleic acid. The conversion of RA to vaccenic acid (*trans*-11 18:1; VA) is catalyzed by a reductase. The structure of VA is given in Figure 3.1 and it is noteworthy that it is an intermediate in the biohydrogenation of both linoleic and linolenic acids (Figure 3.2). The final step is a further reduction of the *trans*-monoenes, producing stearic acid. Reduction of *trans*-octadecenoic fatty acids to stearic acid is generally the rate-limiting step and, as a consequence, there is often an accumulation of *trans* fatty acids in the rumen (Keeney, 1970).

As analytical techniques improved, we have gained an appreciation of the complexity of the biohydrogenation processes occurring in the rumen. In addition to the major pathways involving RA and VA as intermediates, there must be many additional pathways. A remarkable range of *trans* 18:1 and CLA isomers are produced during biohydrogenation and their outflow from the rumen based on limited data from growing cattle and lactating cows are shown in Table 3.2. This range of CLA and *trans*-18:1 isomers is

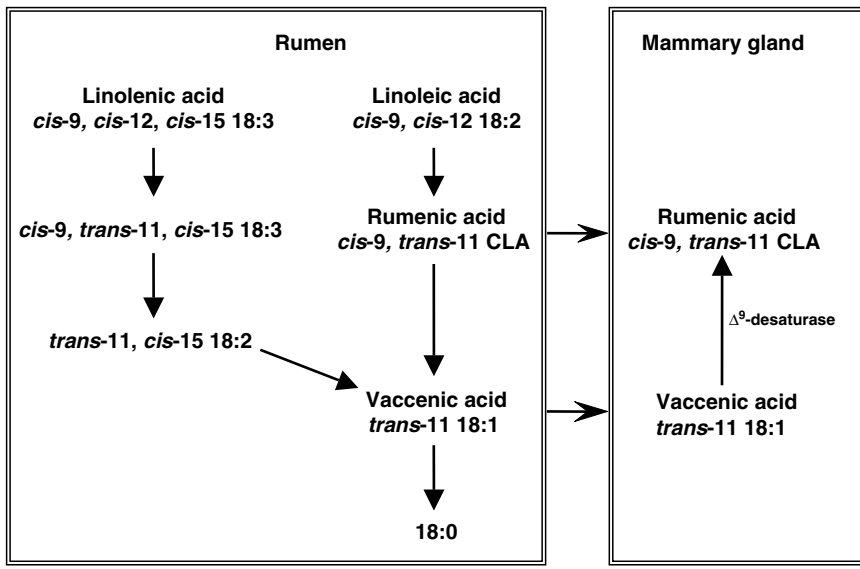

Figure 3.2. Pathways for ruminal and endogenous synthesis of rumenic acid (*cis*-9, *trans*-11 CLA) in the lactating dairy cow. Pathways for biohydrogenation of linoleic and linolenic acids yielding vaccenic acid (*trans*-11 18:1) are shown in the rumen box and endogenous synthesis by Δ^9-desaturase is shown in the mammary gland box. Adapted from Bauman *et al*. (2003).

Table 3.2. Range of double bond positions in *trans* $C_{18:1}$ and conjugated $C_{18:2}$ fatty acids and their ruminal outflow in growing and lactating cattle*

Trans 18:1		Conjugated 18:2	
Isomer	Ruminal outflow (g/day)	Isomer	Ruminal outflow (g/day)
trans-4	0.5–0.7	*trans*-7, *cis*-9	<0.01
trans-5	0.4–0.6	*trans*-7, *trans*-9	<0.01–0.05
trans-6-8	0.4–6.7	*trans*-8, *cis*-10	0.01–0.02
trans-9	0.8–6.2	*trans*-8, *trans*-10	<0.01–0.10
trans-10	1.7–29.1	*cis*-9, *cis*-11	<0.01–0.01
trans-11	5.0–121.0	*cis*-9, *trans*-11	0.19–2.86
trans-12	0.5–9.5	*trans*-9, *trans*-11	0.22–0.55
trans-13 + 14	6.5–22.9	*trans*-10, *cis*-12	0.02–0.32
trans-15	3.2–8.5	*trans*-10, *trans*-12	0.05–0.06
trans-16	3.1–8.0	*cis*-11, *trans*-13	0.01–0.10
		trans-11, *cis*-13	0.01–0.46
		trans-11, *trans*-13	0.09–0.40
		cis-12, *trans*-14	<0.01–0.05
		trans-12, *trans*-14	0.08–0.19

* Data derived from three studies where samples were collected from the duodenum (Duckett *et al*., 2002; Piperova *et al*., 2002) or omasum (Shingfield *et al*., 2003).

not accounted for by known pathways of rumen biohydrogenation. Isomerase is the enzyme that catalyses the key step that introduces the conjugated double bond system and, unfortunately, this enzyme has been studied in only a few species of rumen bacteria (Kepler and Tove, 1967; Kepler *et al*., 1970; Yokoyama and Davis, 1971). The isomerase from *Butyrivibrio fibrisolvens* is a particulate enzyme bound to the bacterial cell membrane and it has an absolute substrate requirement for a *cis*-9, *cis*-12 diene system and a free carboxyl group (Kepler and Tove, 1967; Kepler *et al*., 1970). If the initial isomerization involves the *cis*-12 double bond, then a *cis*-9, *trans*-11 conjugated diene is produced, whereas if the initial double bond isomerized is the *cis*-9, then *trans*-10, *cis*-12 conjugated diene is produced. Most rumen bacteria capable of carrying out this isomerization produce mainly RA from linoleic acid. However, Kim *et al*. (2002) recently demonstrated that the rumen bacterium, *Megasphaera elsdenii* YJ-4, produced predominately *trans*-10, *cis*-12 CLA and only a minor quantity of RA when incubated with linoleic acid. Nevertheless, the extent to which the various pathways of biohydrogenation are associated with specific enzymes and species of bacteria or reflect a general lack of specificity of the bacteria and their enzymes is not known.

It appears that the type of diet, rather than level of intake, is a major factor affecting biohydrogenation, and diet-induced changes in the rumen environment can shift the biohydrogenation pathways resulting in dramatic changes in the fatty acid intermediates. In addition, recent studies have established that the metabolism of radio-labelled oleic and eladic acids by mixed ruminal microorganisms results in extensive labelling of a wide range of *trans* octadecenoic fatty acids (*trans*-6 to *trans*-16) as well as of stearic acid (Mosley *et al*., 2002; Proell *et al*., 2002). In a few cases, the biological implications of the changes in the pathways of rumen biohydrogenation have been established and these will be discussed in later sections. A more comprehensive discussion of lipid metabolism in the rumen and its effects on the production of CLA and *trans* 18:1 isomers is provided in a recent review by Palmquist *et al.* (2005).

3.4.2. *cis*-9, *trans*-11 CLA (Rumenic Acid)

Initially, it was assumed that the RA in milk fat and body fat of ruminants originated from incomplete biohydrogenation in the rumen. This hypothesis was based on the fact that RA was the major CLA isomer in ruminant fat and the first intermediate in the major biohydrogenation pathway for linoleic acid (Figure 3.2). A close linear relationship was also observed between the levels of VA and RA in milk fat (Jiang *et al*., 1996; Jahreis *et al*., 1997; Lawless *et al*., 1998; Griinari and Bauman, 1999), consistent with the concept that these two fatty acid intermediates had escaped complete biohydrogenation in the rumen and were subsequently absorbed from the digestive tract and used for milk fat synthesis. However, there were a number of inconsistencies with this idea. Firstly, the kinetics of rumen biohydrogenation are such that CLA represents only a transitory product, and VA is the major biohydrogenation intermediate that accumulates in the rumen (Keeney, 1970; Harfoot and Hazelwood, 1997). Secondly, nutrition studies demonstrated that increases in the milk fat content of CLA occurred when linseed oil and other dietary sources of linolenic acid were fed (e.g., Kelly *et al*., 1998a; Dhiman *et al*., 2000; Lock and Garnsworthy, 2002). As previously discussed, RA is not an intermediate in the biohydrogenation of linolenic acid, but the biohydrogenation of both linoleic and linolenic acids produces VA as an intermediate. Thirdly, the ratio of VA to RA is >50:1 in rumen fluid but only about 3:1 in milk fat. Based on these considerations, Griinari and Bauman (1999) proposed that endogenous synthesis could be an important source of the RA found in milk fat, with synthesis involving the enzyme Δ^9-desaturase and VA as the substrate (see Figure 3.2). Previous investigations with Δ^9-desaturase from rat liver established that while the preferred reaction was the conversion of stearic acid to oleic acid,

this enzyme could also desaturate positional isomers of *trans*-octadecenoic acids (Mahfouz *et al*., 1980; Pollard *et al*., 1980).

The first study to show directly that milk fat CLA could originate *via* endogenous synthesis was Griinari *et al*. (2000b); they infused 12.5 g/d of VA into the abomasum of dairy cows and observed a 31% increase in the concentration of RA in milk fat. This investigation clearly demonstrated the potential for endogenous synthesis, but additional studies were needed to determine its actual importance. To address this, two approaches have been used; one approach was to inhibit Δ^9-desaturase directly, and this has involved the use of sterculic oil which contains two cyclopropene fatty acids, sterculic acid and malvalic acid, which are specific inhibitors of Δ^9-desaturase (Phelps *et al*., 1965; Bickerstaffe and Johnson, 1972; Jeffcoat and Pollard, 1977). To account for the fact that inhibition of Δ^9-desaturase may not be complete, these investigations employed a correction factor based on the ratio in milk fat of *cis*-9 $C_{14:1}$ to $C_{14:0}$ (another product: substrate pair of Δ^9-desaturase that is almost exclusively synthesized in the mammary gland). Griinari *et al*. (2000b), using this approach, estimated that 64% of the RA in milk fat was of endogenous origin in cows fed an alfalfa hay/corn grain-based diet. This represented the first direct demonstration that endogenous synthesis was the major source of RA in milk fat. Subsequent investigations using the same approach extended results to other dietary situations (total mixed diets with or without plant oils and pasture) and in all cases endogenous synthesis was the predominant source of the RA in milk fat (Corl *et al*., 2001; Kay *et al*., 2004). Results obtained with grazing cows are of special note because pasture is high in linolenic acid and endogenous synthesis accounted for >91% of the total RA in milk fat (Kay *et al*., 2004).

The second approach to quantify the contribution of endogenous synthesis to milk fat CLA was indirect and involved a comparison of ruminal outflow with secretion in milk; rumen output of RA would represent the maximum proportion of RA secreted in milk fat and the remainder would have to be derived from endogenous synthesis. For this approach, representative samples of digesta were obtained and data for CLA content were combined with marker-derived estimates of flow rates of digesta. Lock and Garnsworthy (2002), who conducted the first such investigation, estimated rumen output of CLA in non-lactating cows and then extrapolated results to lactating cattle on the basis of feed intake. Their estimates indicated that endogenous synthesis accounted for over 80% of the RA in milk fat in cows fed a grass silage/concentrate diet supplemented with various plant oils. Comparable results were obtained in subsequent studies using this approach with cows fed corn-silage diets containing either a high or low level of forage (Piperova *et al*., 2002), and grass silage/concentrate diets including fish oil supplements (Shingfield *et al*., 2003). Recently, Palmquist *et al*. (2004)

used a mathematical modelling approach to quantify the importance of endogenous synthesis of CLA in adipose tissue of lambs, but it has not yet been applied to lactating cows.

Overall, investigators using different diets and experimental approaches have found similar results; the major source of RA in milk fat is endogenous synthesis (Figure 3.2). Thus, endogenous synthesis is the basis for *cis*-9, *trans*-11 being the predominant CLA isomer in milk fat and the relatively constant ratio between VA and RA observed in milk fat reflects the substrate: product relationship for Δ^9-desaturase.

3.4.3. *trans*-7, *cis*-9 CLA

Yurawecz *et al.* (1998) were the first to identify the presence of *trans*-7, *cis*-9 CLA in milk fat and to do so they used combinations of silver nitrate-HPLC, GC-MS and Fourier transform infrared spectroscopy. This CLA isomer had not been detected previously because it co-eluted with RA in GC methods that were in routine use. Thus, concentrations of RA reported in the scientific literature typically include *trans*-7, *cis*-9 CLA as a component. Across studies, the level of *trans*-7, *cis*-9 CLA in milk fat has generally been on the order of 10% of RA and several-fold greater than any of the other CLA isomers (Sehat *et al.*, 1998; Yurawecz *et al.*, 1998; Bauman *et al.*, 2000; Corl *et al.*, 2002; Piperova *et al.*, 2002). Early investigations with Δ^9-desaturase from rat liver had established that *trans*-7 18:1 could serve as a substrate for this enzyme (Mahfouz *et al.*, 1980; Pollard *et al.*, 1980). Furthermore, *trans*-7 18:1 is present in rumen outflow, albeit at low concentrations (Table 3.2), being produced as an intermediate in the biohydrogenation of oleic acid and 18-carbon PUFAs, as discussed earlier. Based on this, when Yurawecz *et al.* (1998) initially discovered *trans*-7, *cis*-9 CLA in milk fat, they speculated that it might originate from endogenous synthesis.

A number of the investigators who determined endogenous synthesis of RA also examined the source of *trans*-7, *cis*-9 CLA in milk fat. Corl *et al.* (2002) inhibited the activity of Δ^9-desaturase in lactating dairy cows with sterculic oil as a source of cyclopropene fatty acids and with *trans*-10, *cis*-12 CLA, a specific inhibitor of both activity and gene expression for Δ^9-desaturase (Lee *et al.*, 1998; Bretillon *et al.*, 1999; Baumgard *et al.*, 2000; Park *et al.*, 2000). Their data indicated that the *trans*-7, *cis*-9 CLA in milk fat was "derived almost exclusively from endogenous synthesis *via* Δ^9-desaturase" (Corl *et al.*, 2002). Consistent with this, they also observed that there was no detectable *trans*-7, *cis*-9 CLA in rumen fluid. Piperova *et al.* (2002) used the indirect approach to calculate rumen outflow by combining duodenal content of *trans*-7, *cis*-9 CLA with an estimate of digesta flow. When estimates of rumen output of this CLA isomer were compared to that secreted in milk,

they concluded that "almost the entire [amount of] *trans*-7, *cis*-9 CLA" found in milk fat must be produced postruminally. Thus, results from these two very different approaches were in agreement that the source of *trans*-7, *cis*-9 CLA in milk fat was endogenous synthesis *via* Δ^9-desaturase from ruminally produced *trans*-7 18:1.

3.4.4. The Δ^9-Desaturase Enzyme System

The predominance of endogenous synthesis as the source of RA and *trans*-7, *cis*-9 CLA in milk fat highlights the critical role of Δ^9-desaturase in the biology of CLA. Although referred to as Δ^9-desaturase in this review, this enzyme is also known as stearoyl-CoA desaturase (EC 1.14.19.1) in biochemistry texts because stearic acid is its most common substrate. The oxidative reaction catalyzed by Δ^9-desaturase involves cytochrome b_5, NAD(P)-cytochrome b_5 reductase and molecular oxygen (Figure 3.3). The CoA ester of VA is the substrate for the formation of RA, but the preferred substrates for Δ^9-desaturase are stearoyl-CoA and palmitoyl-CoA, which are converted to oleoyl-CoA and palmitoleoyl-CoA, respectively (Ntambi, 1999). For ruminants, a substantial activity of Δ^9-desaturase has been reported in mammary tissue (Bickerstaffe and Annison, 1970; Kinsella, 1972; McDonald and Kinsella, 1973; Wahle, 1974), adipose tissue (Wahle, 1974; Chang *et al.*, 1992; Cameron *et al.*, 1994; Barber *et al.*, 2000) and in intestinal epithelium (Bickerstaffe and Annison, 1969). In contrast to rodents, the ruminant liver has only negligible activity. Both bovine (Cooney and Headon, 1989; Chung *et al.*, 2000) and ovine (Ward *et al.*, 1998) Δ^9-desaturase genes have been cloned and only one gene has been found. This is similar to humans, but differs from rodents where two isoforms of the gene have been identified in rats and three isoforms of the gene have been characterized in mice (Ntambi and Miyazaki, 2004).

Our understanding of the regulation of Δ^9-desaturase in ruminants is limited, with current knowledge coming mainly from investigations on

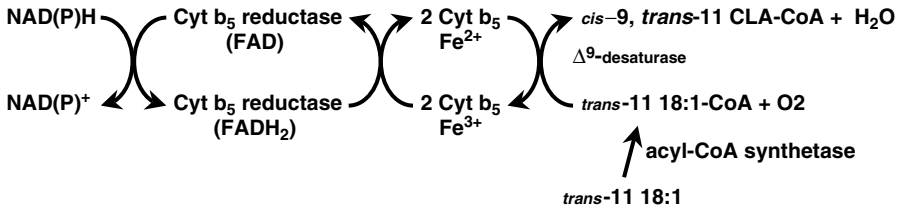

Figure 3.3. The Δ^9-desaturase enzyme system showing the conversion of vaccenic acid (*trans*-11 18:1) to rumenic acid (*cis*-9, *trans*-11 CLA).

rodents. Δ^9-Desaturase has no known allosteric or feedback inhibition involving its substrates or products. However, it is regulated by dietary factors such as glucose and PUFAs, and by hormones such as insulin and glucagon (Ntambi and Miyazaki, 2004). The enzyme protein has a relatively short half-life ($\sim$4 h) and thus gene transcription is its major point of regulation (Ozols, 1997). Both PUFAs and *trans*-10, *cis*-12 CLA down-regulate gene expression, but RA has no effect (Lee *et al*., 1998; Choi *et al*., 2000; Ntambi and Miyazaki, 2004). Interestingly, the cyclopropene fatty acids in sterculic oil do not affect expression of the Δ^9-desaturase gene or protein, but they directly inhibit the activity of the enzyme (Gomez *et al*., 2003).

At a cellular level, regulation of Δ^9-desaturase in mammary tissue appears to involve the sterol-response-element-binding-protein (SREBP) family of transcription factors (Peterson *et al*., 2004). PUFAs inhibit the processing of SREBP-1 and may decrease the abundance of the precursor protein, leading to reduction in transcription of many genes in the lipogenic pathways, including Δ^9-desaturase (Shimano, 2001; Horton *et al*., 2002). Ward *et al*. (1998) reported high expression of Δ^9-desaturase mRNA in adipose tissue and mammary gland of lactating sheep, and expression was decreased by 80% in adipose tissue of animals during pregnancy and lactation, a time when lipogenic activity is increased in mammary gland and decreased in adipose tissue (Bauman and Currie, 1980).

The relationship between substrate and product for Δ^9-desaturase is reflected by the desaturase index, defined as [RA $\div$ (RA + VA)] (Kelsey *et al*., 2003). The desaturase index in milk fat represents a proxy for Δ^9-desaturase and a several-fold range is observed among individuals. This is discussed in Section 3.5.2, but provides a strong indication that there are genetic differences among individuals with respect to this enzyme.

3.4.5. Other CLA Isomers

In contrast to *cis*-9, *trans*-11 and *trans*-7, *cis*-9, the other isomers of CLA found in the milk fat of ruminants appear to originate exclusively from rumen output. This conclusion is based, in large part, on the fact that these minor *cis-trans*, *trans-cis*, *cis-cis*, and *trans-trans* isomers are detected in rumen fluid (Corl *et al*., 2002) and duodenal fluid (Piperova *et al*., 2002; Shingfield *et al*., 2003), and estimates of digesta flow indicate that rumen output is more than adequate to account for the trace amounts secreted in milk fat (Piperova *et al*., 2002; Shingfield *et al*., 2003). Furthermore, there has been no demonstration that other mammalian desaturases act in a manner analogous to Δ^9-desaturase to synthesize CLA endogenously from *trans* octadecenoic fatty acids. Thus, these CLA isomers found at trace levels

in milk fat are logically of rumen origin and represent intermediates formed in the biohydrogenation of PUFAs.

Information on the effect of diet on the production of minor isomers of CLA in the rumen and alterations in their content in milk fat is limited. Diet-induced changes in *trans*-10, *cis*-12 CLA have been best described, and its biological effects in the dairy cow will be discussed in Section 3.6.1. Griinari and Bauman (1999) presented a putative pathway for the biohydrogenation of linoleic acid where the initial isomerization involved the *cis*-9 double bond, thereby resulting in the production of *trans*-10, *cis*-12 CLA and *trans*-10 18:1 as intermediates. As discussed earlier, rumen bacteria have been identified that produce *trans*-10, *cis*-12 CLA when incubated with linoleic acid (Verhulst *et al*., 1987; Kim *et al*., 2002), and the addition of *trans*-10, *cis*-12 CLA to the rumen results in the increased formation of *trans*-10 18:1 (Loor and Herbein, 2001).

Diet has also been shown to influence rumen output and milk fat content of other minor CLA isomers, although they always remain a small portion of total CLA in milk fat. For example, dietary supplements rich in linolenic acid increased the relative proportions of *trans*-11, *cis*-13 CLA, *trans*-11, *trans*-13 CLA, *cis*-12, *trans*-14 CLA, and *trans*-12, *trans*-14 CLA (Griinari *et al*., 2000a; Griinari and Shingfield, 2002). Ruminal output of *trans*-*trans* CLA isomers with double bonds at positions 9, 11 and 10, 12 was enhanced when diets contained high amounts of concentrates (Piperova *et al*., 2002) or were supplemented with fish oil (Shingfield *et al*., 2003). Recently, Kraft *et al*. (2003) reported that *trans*-11, *cis*-13 CLA represented 2 to 8% of total CLA in milk fat from cows grazing in the Alps. Kramer *et al*. (2004) verified this when they found relatively high concentrations of this isomer in a cheese sample produced from the milk of Alpine cows and *trans*-11, *cis*-13 CLA was also observed at significant concentrations in a sample of Yak milk fat (Cruz-Hernandez *et al*., 2004). Kraft *et al*. (2003) suggested that the *trans*-11, *cis*-13 CLA might be produced *via* rumen biohydrogenation of linolenic acid based on the lipid content of Alpine pasture, but this has not been established directly. Overall, most investigations of the effects of diet on CLA have not used the detailed analytical methods required to resolve the full range of minor CLA isomers. Thus, more completely identifying rumen biohydrogenation pathways and establishing their relationship to specific rumen bacteria and diets are important areas for future research.

3.5. Modification of CLA Content in Milk Fat

The discovery of health benefits of CLA and a recognition of the potential of RA as a functional food component in dairy products has stimulated research to identify factors that affect the CLA content of milk fat. These

efforts have focused on enhancing the CLA content per unit of fat and centered on RA as the predominant CLA isomer. From the preceding discussion on the origin of RA (Section 3.4.2), there are four possibilities to consider: (i) increase the 18-carbon PUFA precursors in the diet (linoleic and linolenic acids); (ii) maintain rumen biohydrogenation pathways that result in the production of VA as an intermediate; (iii) inhibit the final step in the biohydrogenation of 18-carbon PUFAs so that VA accumulates; and (iv) increase Δ^9-desaturase and the desaturation of VA to RA in the mammary gland. In the following sections, we will discuss results of investigations designed to establish dietary and nutritional conditions that maximize rumen outflow of VA and RA, optimize the amount and activity of Δ^9-desaturase in mammary tissue, and identify the physiological basis for the large differences among individuals in terms of the production of CLA. Obviously, before CLA-enriched foods are widely marketed, effects on quality and consumer acceptability of the dairy products also need to be examined, and the limited research on this will be summarized also.

3.5.1. Dietary and Nutritional Effects

Numerous studies have shown that diet is the most significant factor affecting the CLA content of milk fat, and its concentration can be increased several-fold by dietary means (see reviews by Chilliard *et al*., 2000; 2001; Bauman *et al*., 2001; Stanton *et al*., 2003; Lock and Bauman, 2004). As cited above, one key to increasing milk CLA is to increase the dietary intake of 18-carbon PUFAs, thereby providing more substrate for rumen biohydrogenation. The dietary supply of linoleic and linolenic acids is most easily increased by the addition of plant oils rich in these fatty acids, and a number of plant oils have been investigated and shown to be effective in increasing the level of CLA in milk fat. For example, dietary supplements of soybean, sunflower, rapeseed or linseed oils have been used successfully to increase the level of CLA in milk fat (Kelly *et al*., 1998a; Dhiman *et al*., 2000; Chouinard *et al*., 2001; Lock and Garnsworthy, 2002).

The slow release of PUFAs in the rumen typically creates favourable conditions for the accumulation of *trans*-18:1 fatty acids, thereby increasing rumen output of VA (Bauman *et al*., 2001). In this regard, the coat of oil seeds offers some protection against rumen biohydrogenation and thus, the use of different oil seeds and processing techniques have been investigated also; correspondingly, a range of oilseeds containing both linoleic and linolenic acids have been shown to be affective in increasing the CLA content of milk fat (e.g., Stanton *et al*., 1997; Dhiman *et al*., 2000; AbuGhazaleh *et al*., 2001; Chouinard *et al*., 2001). In general, oil seeds that are rich in 18-carbon PUFAs

and processed so that the oil is accessible to the bacteria involved in biohydrogenation result in greater increases in milk CLA compared with whole oil seeds, but are not as efficient as using the pure oil (Lock and Bauman, 2004). The use of calcium salts of fatty acids derived from plant oils has also been investigated because of the partial protection that the calcium-fatty acid complex offers from rumen biohydrogenation. Chouinard *et al.* (2001) fed calcium salts of fatty acids derived from rape, soybean and linseed oils; all three increased the CLA content of milk fat, with the largest increases occurring for those containing the greatest amounts of linoleic and linolenic acids (soybean and linseed, respectively).

The amount of 18-carbon PUFAs that can be added to the diets of dairy cows is limited due to the adverse effects these PUFAs can have on the metabolism of rumen bacteria, thereby impairing rumen fermentation and animal performance (Jenkins, 1993). Thus, dairy cattle diets are generally restricted to less than 7% total lipid, and this provides an upper limit to the use of lipid supplements. Oilseeds and chemical protection of oils offer some benefit as they often allow for greater amounts of the oil to be fed before negative effects on microbial growth and metabolism are realized. When a high level of oil is added, up to 10-fold increases in the CLA content of milk fat are observed, but because of negative effects on rumen bacteria discussed above, these levels are often transient and decline within a few weeks to stabilize at ~4-fold to 5-fold increases (e.g., Bauman *et al.*, 2000). In addition, there is often a fine line between supplying additional lipid supplements to increase milk fat CLA content and causing changes in the rumen environment; for example, under some conditions the rumen environment may be modified to produce more *trans*-10 18:1 and *trans*-10, *cis*-12 CLA as intermediates and this results in a dramatic reduction in milk fat synthesis (see Section 3.6.1).

Another means through which dietary and nutritional factors can increase the CLA content of milk fat is by inhibiting the terminal step in biohydrogenation (Figure 3.2). This typically occurs either directly or indirectly *via* changes in the rumen environment; the net result is an accumulation of VA, thereby increasing the rumen outflow of this precursor for the endogenous synthesis of CLA. A limited number of bacterial species have been shown to carry out the final biohydrogenation step and, presumably, changes in the rumen environment lead to a reduction in these species and/or a reduction in their capacity to reduce VA to stearic acid. Several dietary situations also have these effects and they include alterations in the forage: concentrate ratio, dietary supplements of fish oil and restricted feeding (see Bauman *et al.*, 2001). The most consistently effective of these is the use of fish oils. Fish oils themselves provide very little 18-carbon PUFAs precursors to allow for increased rumen VA output, indicating that this increase occurs

through an inhibition of the biohydrogenation of VA; indeed, C22:6 n-3 (docosahexaenoic acid, DHA), a major n-3 fatty acid in fish oil, has been shown to promote the accumulation of VA in mixed ruminal cultures when incubated with linoleic acid (AbuGhazaleh and Jenkins, 2004). Both linoleic and linolenic acids are plentiful in forages and concentrates which provide sufficient 18-carbon PUFA precursors. A range of fish and marine oils have been used with success, and similar to the supply of 18-carbon PUFAs discussed above, both lipid supplements and fish by-products (fish meal) have been shown to be effective (e.g., Offer *et al*., 1999; Donovan *et al*., 2000; AbuGhazaleh *et al*., 2001; Shingfield *et al*., 2003). Marine algae also contain long chain PUFAs and have also been effective (Franklin *et al*., 1999).

The most effective dietary treatments for increasing the CLA content of milk fat are those that both increase the supply of 18-carbon PUFAs and modify the rumen environment. The most widely studied of these is the use of fresh pasture, with numerous studies indicating that fresh pasture results in a 2-fold to 3-fold increase in the CLA content of milk fat (e.g., Stanton *et al*., 1997; Kelly *et al*., 1998b; Dhiman *et al*., 1999). The degree of response, however, decreases as the pasture matures and the proportion in the diet decreases. Correspondingly, seasonal effects on milk CLA content have been reported, with the trend that the content is greatest when fresh pasture is plentiful, and decreases throughout the growing season (Riel, 1963; Banni *et al*., 1996; Auldist *et al*., 2002; Lock and Garnsworthy, 2003). These results cannot be explained fully in terms of the fatty acid composition and supply of PUFAs that grass provides; therefore, there must be additional factors or components of grass that promote the production of VA in the rumen, and these lessen in effect as the pasture matures (Lock and Bauman, 2004). Presumably, these factors inhibit the conversion of VA to stearic acid, as discussed previously. The effect of different farming systems has also been investigated, with systems differentiated by the amount and type of forage typically fed to cows. In general, production systems with the greatest proportion of fresh forage in the diet give the highest level of CLA in milk fat. For example, Jahreis *et al*. (1997) reported that cows grazed during the summer months had a higher level of CLA in milk than cows housed all-year round and fed conserved forage.

Although the use of fresh pasture has striking effects on enhancing the CLA content of milk fat, a similar increase is possible using standard dietary ingredients such as plant oils/oilseeds and fish oil/fish meal supplements. Further, there is some indication that dietary regimes involving a combination of supplements can have an additive effect on increasing the level of CLA in milk; for example Whitlock *et al*. (2002) observed higher levels with a combination of plant oil and fish oil than when either was fed alone. In all of the dietary situations designed to enhance the level of CLA in milk fat, it

is vital that the normal VA pathway of biohydrogenation is maintained. If shifts in biohydrogenation occur, then the pattern of *trans* fatty acids changes and there will be a reduction in the rumen output of VA, and as a consequence a reduction in the level of CLA in milk fat. This shift in the pathways of biohydrogenation is also associated with an increased risk of depression of milk fat synthesis (see Section 3.6.1).

3.5.2. Physiological Factors

Physiological factors also have an impact on the content of CLA in milk fat. Surveys have shown an 8-fold range in the milk fat content of CLA among herds (Riel, 1963; Kelly and Bauman, 1996) and these differences in large part reflect diet and nutritional effects as discussed above. However, substantial differences are observed among cows within a herd consuming the same diet. Investigations involving diets ranging from corn-based total mixed rations to pasture have all shown a 2-fold to 3-fold range in the milk fat content of CLA among individual cows (e.g., Kelly *et al.*, 1998a, b; Lawless *et al.*, 1998; Lock and Garnsworthy, 2002; 2003; Peterson *et al.*, 2002b). Thus, across diets that result in substantial differences in the average milk fat content of CLA, a similar 2-fold to 3-fold range is observed among cows consuming the same diet. This variation would in large part be related to individual differences in, (1) rumen output of VA and to a lesser extent CLA, and (2) the amount and activity of Δ^9-desaturase.

The final method to enhance the level of CLA in milk is to increase endogenous synthesis and this probably explains the variation among cows in a herd fed the same diet. Undoubtedly, the variation in Δ^9-desaturase among individuals has a genetic basis (Bauman *et al.*, 2003), but this has not been examined directly. However, an indirect evaluation is possible because milk fat contains four major fatty acid pairs that represent a product/substrate relationship for Δ^9-desaturase, myristoleic/myristic acid, palmitoleic/palmitic acid, oleic/stearic acid and RA/VA. Ratios for these pairs of fatty acids, referred to as a desaturase index, represent a proxy for Δ^9-desaturase activity. In the largest study to examine this hypothesis, Kelsey *et al.* (2003) found that the variation in milk fat content of RA and the desaturase index was about 3-fold among individuals consuming the same diet. Other investigators have also observed a 2-fold to 3-fold range in desaturase index among cows in the same herd (Lock and Garnsworthy, 2002; 2003; Peterson *et al.*, 2002b). Peterson *et al.* (2002b) also demonstrated a consistency in the individual hierarchy in desaturase index over time when cows were fed the same diet and a consistency in the individual hierarchy when cows were switched between diets. Presumably, this variation reflects individual differences in the activity of Δ^9-desaturase involving the regulation of gene expression, primary

or tertiary structure of the enzyme due to gene polymorphisms, post-translational modifications, or other factors affecting the interaction between the enzyme and the substrate or product.

Several specific physiological factors have been examined for effects on the level of CLA in milk fat, but because of the large impact of diet and the wide range among individuals, it is important that these comparisons involve a reasonable number of cows fed a common diet. These conditions were met in the studies by Kelsey *et al.* (2003) and Lock *et al.* (2005a), and both found that the CLA content of milk fat and the desaturase index had no relationship to parity or stage of lactation (days in milk). Likewise, they observed that the milk fat content of CLA and desaturase index also had no relationship to milk yield, milk fat percentage or yield of milk fat (Kelsey *et al.*, 2003; Lock *et al.*, 2005a). The investigation by Kelsey *et al.* (2003) involved over 200 cows fed the same diet and showed no difference between Holstein and Brown Swiss breeds. In contrast, several studies have reported breed differences in the CLA content of milk fat (Lawless *et al.*, 1999; White *et al.*, 2001; Whitlock *et al.*, 2002), which may reflect differences in desaturase index among breeds. However, these studies often involved very few animals or were confounded by diet, or both. Using a larger data set, DePeters *et al.* (1995) reported breed differences in the desaturase index in the milk fat of dairy cows, consistent with the suggestion that the activity of Δ^9-desaturase is higher in Holstein than in Jersey mammary tissue (Beaulieu and Palmquist, 1995). However, if breed differences exist they would appear to be minor compared with the magnitude of dietary effects and variation among cows in terms of both the CLA content of milk fat and desaturase index.

Increasing Δ^9-desaturase activity would not only impact on the level of CLA in milk fat, but would also increase other unsaturated fatty acids that are products of this enzyme. As a consequence of these changes, the saturated:unsaturated content of milk fat would be altered resulting in an improvement in the "human health" characteristics of milk fat. Thus, establishing the heritability of individual differences in the desaturase index and the extent to which this could be used in genetic selection programmes is of interest. This potential to improve the fatty acid composition of milk fat is also the basis for recent work to produce transgenic goats that have greater expression of Δ^9-desaturase in the mammary gland (Reh *et al.*, 2004).

3.5.3. Manufacturing and Product Quality Considerations

Consumer surveys indicate an interest in dairy products that are enriched in CLA (Ramaswany *et al.*, 2001b). As outlined in preceding sections, the level of CLA in milk fat can be enhanced several-fold naturally by diet formulation and selection of individual cows with elevated milk fat CLA.

But central to marketing and consumer acceptance of CLA-enriched foods is a consideration of the effects of processing and storage, and the final sensory characteristics of CLA-enriched products. Many dairy products undergo a microbial fermentation during processing and the effects of these on the CLA content have been of special interest. Several studies have investigated this and found that food processing and manufacturing have little or no effect on CLA content (Shantha *et al*., 1992, 1995; Werner *et al*., 1992; Jiang *et al*., 1997; Lin *et al*., 1999: Gnädig *et al*., 2004). As emphasized in the review by Parodi (2003), any changes in the CLA content related to processing or to storage are minimal when compared to the variations associated with diet formulations and differences between individual cows. Thus, the final concentration of CLA in dairy products is, in large part, related to the CLA concentration in the raw milk fat and the fat content of the final product.

Consumer acceptability of CLA-enriched dairy products is also dependent on their taste and organoleptic properties. Off-flavours due to fatty acid oxidation are of prime concern because diet formulation methods used to enhance milk fat with respect to CLA generally cause an increase in the proportion of unsaturated fatty acids in the milk fat (Lock and Bauman, 2004). Reports on sensory characteristics and quality of naturally-enriched dairy products, typically having a 2-fold to 3-fold increase in milk fat CLA, have generally indicated no differences from unenriched dairy products (Ramaswamy *et al*., 2001a, b; Baer *et al*., 2001; Avramis *et al*., 2003; Gonzalez *et al*., 2003). An exception is Lacasse *et al*. (2002) who found that 2.7%-fat milk from cows fed either protected (3% of dry matter) or unprotected fish oil (3.7% of dry matter) scored significantly lower in flavor and taste. However, the levels of fish oil used in this study were significantly greater than used by others.

Lynch *et al*. (2005) compared the flavor, organoleptic and storage characteristics of standard 2%-fat milk with 2%-fat milk that had an approximately 10-fold higher level of CLA. The naturally enhanced milk (the level of CLA and VA was 47 and 121 mg/g fatty acids, respectively) was produced through individual selection and nutritional management of the cows. Initial evaluation of the milk and evaluation over a 14-day post-pasteurization period indicated no flavor differences as determined by triangle taste tests. Similarly, sensory results indicated no differences in susceptibility to the development of oxidized off-flavors between the control and CLA-enhanced milks, even when milk was stored under light (Lynch *et al*., 2005). Thus, flavor and consumer acceptability were maintained in a dairy product with substantially enhanced levels of CLA and VA.

The research discussed above involved dairy products that were naturally enriched with CLA through formulation of diets known to increase

the level of CLA in milk fat and selection of individual cows with a higher level of CLA in their milk fat. Campbell *et al.* (2003) used an alternative approach involving fortification of milk fat with synthetic CLA during the manufacturing process. They added 1 or 2% CLA-containing triglycerides to skim milk together with vitamin E and rosemary extract to retard lipid oxidation. Descriptive sensory analysis revealed that the fortified milk had a ''grassy/vegetable oil'' flavor and consumer acceptability scores were low, although acceptability was improved when a chocolate flavor was added.

Overall, results to date indicate that manufacturing and quality characteristics were normal for dairy products naturally enriched with CLA and consumer acceptability was comparable to unenriched dairy products. However, the single study examining fortification of skim milk with synthetic CLA during the manufacturing process had poor consumer acceptability.

3.6. Biological Effects of CLA Isomers

A broad overview of the biological effects of CLA is presented elsewhere in this volume (Chapter 17), so the emphasis in the following section will be two-fold. Firstly, the biology of *trans*-10, *cis*-12 CLA in the dairy cow will be summarized because under certain dietary conditions, production of this isomer in the rumen can profoundly affect milk fat synthesis. Secondly, the biological effects of RA when supplied as a natural component of the diet will be reviewed because this CLA isomer represents a functional component of milk fat that has potential health benefits. Although other CLA isomers are present in milk fat, they are present at concentrations much too low to have a significant effect.

3.6.1. *trans*-10, *cis*-12 CLA and Lipid Metabolism

3.6.1.1. Inhibition of Milk Fat Synthesis

Investigations in which the transfer of CLA to milk fat in dairy cows was examined showed that supplementation of mixed isomers of CLA resulted in a dramatic reduction in milk fat secretion (Loor and Herbein, 1998; Chouinard *et al.*, 1999a, b). Decreases of up to 50% in milk fat yield occurred and the effects were reversed when supplementation was terminated. Furthermore, effects were specific for milk fat with the yield of milk and other milk components being relatively unaffected. Initial investigations were of short duration (<7 days) and the CLA supplement was infused abomasally as a convenient experimental method to avoid possible alterations during rumen fermentation. However, subsequent long-term studies (20 weeks) demonstrated that the reduction in milk fat synthesis was

maintained when a rumen-protected formulation of CLA was used (Perfield *et al*., 2002; Bernal-Santos *et al*., 2003).

Early investigations utilized CLA supplements that were composed of a mixture of, generally, four or more isomers. Baumgard *et al*. (2000) reported the first evidence of the differential effect of specific CLA isomers on milk fat synthesis; they demonstrated that abomasal infusion of *trans*-10, *cis*-12 CLA resulted in an immediate decrease in milk fat synthesis whereas RA had no effect. Recently, additional CLA isomers have been examined *via* abomasal infusion and these have included *trans*-8, *cis*-10 CLA, *cis*-11, *trans*-13 CLA, and *trans*-10, *trans*-12 CLA (Perfield *et al*., 2004a, c). Although all of these isomers were taken up by the mammary gland and incorporated into milk fat, none affected the rate of milk fat synthesis. Thus, *trans*-10, *cis*-12 CLA is the only CLA isomer that has been shown to reduce milk fat synthesis. The initial step in the metabolism of linoleic and linolenic acids to form eicosanoids is catalyzed by Δ^6-desaturase. The metabolite formed by the action of Δ^6-desaturase on *trans*-10, *cis*-12 CLA is *cis*-6, *trans*-10, *cis*-12 18:3. Investigations of this fatty acid, as well as *cis*-6, *trans*-8, *cis*-12 18:3, have established that neither of these conjugated trienoic 18:3 fatty acids affect milk fat synthesis or any other lactational variable (Sæbø *et al*., 2005).

Relationships between *trans*-10, *cis*-12 CLA and milk fat synthesis have been examined. There is a curvilinear relationship between the reduction in milk fat yield and the abomasal infusion dose of *trans*-10, *cis*-12 CLA (Figure 3.4). *Trans*-10, *cis*-12 CLA is a very potent inhibitor of milk fat synthesis in dairy cows; a dose of 2.0 g/d (<0.01% of dry matter intake) reduced milk fat synthesis by 20%. *Trans*-10, *cis*-12 CLA is also incorporated into milk fat and in this case the relationship is linear (Figure 3.4); a summary of seven studies showed that the transfer efficiency of abomasally-infused *trans*-10, *cis*-12 CLA into milk fat averaged 22% (de Veth *et al*., 2004). The linear relationship in transfer to milk fat is remarkable when one considers that the yield of milk fat is simultaneously decreased as the abomasal dose of *trans*-10, *cis*-12 CLA is increased. This suggests that the mechanisms which coordinate the CLA-induced decrease in the use of preformed fatty acids for milk fat synthesis have a less pronounced effect on the mammary uptake and incorporation of *trans*-10, *cis*-12 CLA into milk fat, but the basis for this difference is unknown.

Initial studies on CLA showed that the reduction in milk fat secretion reflected decreases in fatty acid levels of all chain-length, but effects were most pronounced for those synthesized *de novo* (Loor and Herbein, 1998; Chouinard *et al*., 1999a,b). As investigations focused on *trans*-10, *cis*-12 CLA and expanded to include a range of doses, it was discovered that at lower doses, the reduction in milk fat was distributed more uniformly among the fatty acids synthesized *de novo* (short-chain and medium-chain length)

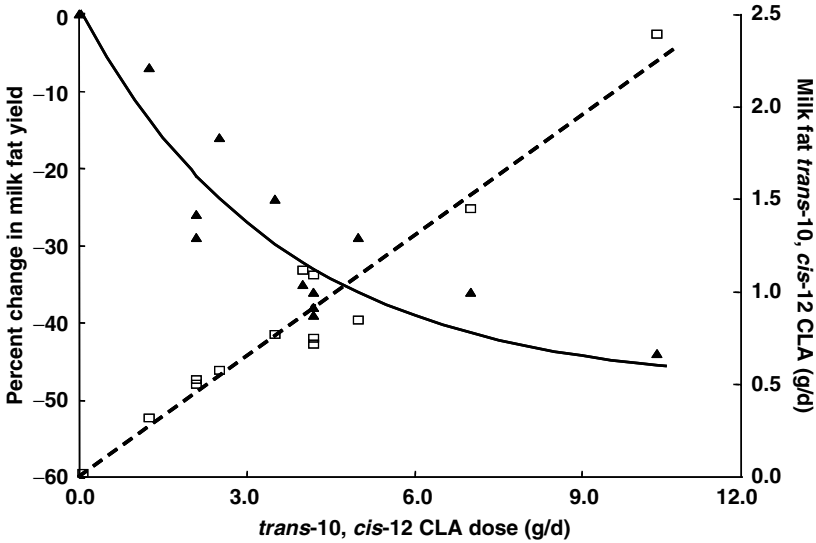

Figure 3.4. Relationships between dose of *trans*-10, *cis*-12 CLA infused into the abomasum and (i) change in milk fat yield (▲; $y = -48.26 + 49.03\exp^{-0.2782x}$; $R^2 = 0.86$), and (ii) secretion of *trans*-10, *cis*-12 CLA into milk fat (□; $y = 0.2175x + 0.0111$; $R^2 = 0.94$). Adapted from a summary by de Veth *et al.* (2004) using data from Baumgard *et al.* (2000, 2001, 2002), Peterson *et al.* (2002a), Sæbø *et al.* (2005), de Veth *et al.* (2004) and Perfield *et al.* (2004c).

and the longer-chain fatty acids taken up from the blood (Baumgard *et al.*, 2001; Peterson *et al.*, 2002a). Likewise, an inhibition of Δ^9-desaturase which resulted in a marked shift in the fatty acid composition of milk fat was observed only at doses of *trans*-10, *cis*-12 CLA where milk fat production was reduced by >20%. At lower doses of *trans*-10, *cis*-12 CLA, the ratio of fatty acids representing product/substrate for Δ^9-desaturase was unaffected (Baumgard *et al.*, 2001; Peterson *et al.*, 2002a; de Veth *et al.*, 2004).

The changes observed in the fatty acid composition of milk in CLA-supplemented cows suggest that many of the processes involved in milk fat synthesis must be affected. Baumgard *et al.* (2002) conducted the first investigation of this by quantifying the abundance of mRNA for several lipogenic enzymes in mammary tissue obtained 5 days after treatment with *trans*-10, *cis*-12 CLA. They found that the 48% reduction in milk fat yield corresponded to a reduction of similar magnitude in the abundance of mRNA for genes that encoded for enzymes involved in the uptake and transport of circulating fatty acids (lipoprotein lipase and fatty acid-binding protein), *de novo* fatty acid synthesis (acetyl CoA carboxylase and fatty acid synthase), desaturation of fatty acids (Δ^9-desaturase), and triglyceride

synthesis (glycerol phosphate acyltransferase and acylglycerol phosphate acyltransferase). Subsequent work using a bovine mammary epithelial cell line has given similar results when cells were incubated with *trans*-10, *cis*-12 CLA (Peterson *et al*., 2004).

The biochemical responses described above support the hypothesis that the reduction in the production of milk fat involves a coordinated regulation of key lipogenic enzymes in the mammary gland, and logical candidates as a central regulator of lipid synthesis are the sterol-response-element-binding-proteins (SREBP). The role of SREBP in the regulation of lipid metabolism has been characterized elegantly in rodents where promoters for sterol response elements have been identified in genes for key enzymes in the pathways of fatty acid synthesis and metabolism (see reviews by Shimano, 2001; Horton *et al*., 2002). Recently, Peterson *et al*. (2004) found that bovine mammary epithelial cells also contain SREBP. They demonstrated further that *trans*-10, *cis*-12 CLA reduced lipid synthesis in these cells through inhibition of the proteolytic activation of SREBP-1 and subsequent reduction in translational activation of lipogenic genes. Thus, the mechanism whereby *trans*-10, *cis*-12 CLA affects milk fat synthesis appears to involve alterations in the activation of this transcription factor.

3.6.1.2. Relationship to Diet-Induced Milk Fat Depression

Under particular dietary situations, a reduction in the content and yield of milk fat occurs in dairy cows. This has commonly been referred to as milk fat depression (MFD) and recent investigations indicate that this metabolic syndrome is related, at least in part, to effects of specific CLA isomers on rates of milk fat synthesis. First described over a century ago, MFD has most often been observed with diets that are low in roughage and high in starch, diets containing plant or fish oil supplements and diets where effective fiber is reduced by processing the forage (e.g., grinding or pelleting). Effects are specific for milk fat and decreases of up to 50% have been observed.

Diet-induced MFD has been the subject of extensive research, especially over the last 50 years (see reviews by Davis and Brown, 1970; Palmquist *et al*., 1993; Bauman and Griinari, 2001). Many theories have been advanced to explain diet-induced MFD, however, most have been found inadequate to explain the cause and mechanism of this phenomenon (Doreau *et al*., 1997a; Bauman and Griinari, 2001, 2003). A shift in rumen fermentation is clearly involved and the occurrence corresponds to a marked increase in the *trans* 18:1 content of milk fat (Davis *et al*., 1970; Griinari *et al*., 1998). While VA is generally the principal *trans* 18:1 isomer in milk fat, a key development was the discovery by Griinari *et al*. (1998) that the change

with diet-induced MFD specifically involved an increase in the *trans*-10 18:1 isomer. Subsequently, this was verified for other dietary conditions (Piperova *et al*., 2000; Offer *et al*., 2001; Peterson *et al*., 2003), and it established that diet-induced MFD involved a shift in the rumen pathways of biohydrogenation, as indicated in Figure 3.5.

Bauman and Griinari (2001) recognized the central role of rumen biohydrogenation in MFD and proposed that "under certain dietary conditions, the pathways of rumen biohydrogenation are altered to produce unique fatty acid intermediates which are potent inhibitors of milk fat synthesis." This is referred to as the "biohydrogenation theory" and results have demonstrated that diet-induced MFD is generally correlated with the level of *trans*-10, *cis*-12 CLA in milk fat (Bauman and Griinari, 2001; Peterson *et al*., 2003; Piperova *et al*., 2004). Further, Bauman and Griinari (2001) suggested that additional unique biohydrogenation intermediates that inhibit fat synthesis may be produced under dietary conditions causing MFD and recent work has offered further support for this idea (Perfield *et al*., 2002; Peterson *et al*., 2003; Piperova *et al*., 2004). The level of *trans*-10 18:1 in milk fat is also highly correlated with the onset of diet-induced MFD, but to date there have been no investigations establishing a direct effect of this fatty acid (see discussion by Bauman and Griinari, 2003). Thus, at this time, the *trans*-10, *cis*-12 CLA isomer is the only rumen biohydrogenation intermediate shown to inhibit the synthesis of milk fat.

Recent investigations of the mechanism of diet-induced MFD indicate that it involves a coordinated reduction in the abundance of mRNA for key

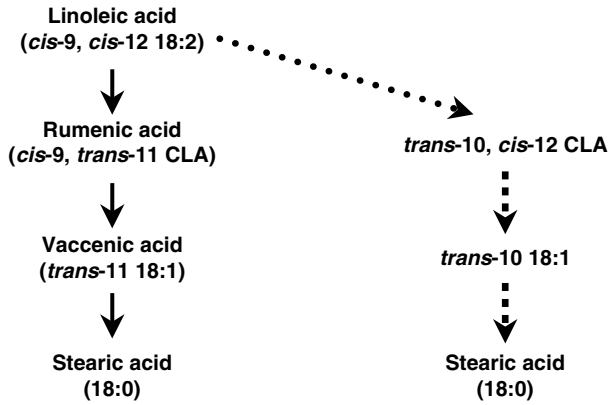

Figure 3.5. Generalized scheme of ruminal biohydrogenation of linoleic acid under normal conditions (solid line) and during diet-induced milk fat depression (dotted line). Adapted from Griinari and Bauman (1999).

enzymes involved in the pathways of milk fat synthesis (Piperova *et al*., 2000; Ahnadi *et al*., 2002; Peterson *et al*., 2003). Thus, mechanisms appear to be identical to those discussed earlier to explain the reduction in the production of milk fat observed with dietary supplementation with *trans*-10, *cis*-12 CLA. Overall, diet-induced MFD represents a natural situation where the production of *trans*-10, *cis*-12 CLA, and probably other unique biohydrogenation intermediates in the rumen, results in a decrease in mammary synthesis of fatty acids and a reduction in milk fat secretion. As knowledge of the biology of CLA increases, comparisons with the physiology of diet-induced MFD will continue to be of interest.

3.6.1.3. Use as a Management Tool

Dietary supplementation with CLA to reduce milk fat yield has potential use as a management tool in milk production. Milk fat is the major "cost" of milk synthesis accounting for over one-half of the energy needed for milk synthesis; consequently, a reduction in milk fat output will result in a sparing of energy that can be used for other purposes. Commercial situations where this could have application include markets where production is regulated by a quota system based on milk fat, and nutritional situations where cows cannot consume sufficient energy to meet their requirements. Examples of the latter include the onset of lactation and the early lactation period, and under adverse environmental conditions such as heat stress or weather-related feed shortages (see Griinari and Bauman, 2003).

Commercial application of *trans*-10, *cis*-12 CLA as a management tool requires a CLA formulation that must have two characteristics; it must offer protection of the CLA from alterations by rumen bacteria and the CLA must subsequently become available for absorption in the small intestine. The majority of protection methods are pH-dependent and take advantage of the transition occurring between rumen pH (~5.8 to 6.7) and pH of the abomasum (~2 to 4). To date, the majority of research on rumen-protected CLA has used supplements consisting of calcium salts of free fatty acids. Perfield *et al*. (2002) used this formulation in the first long-term investigation using cows in late lactation; they observed that the reduction in the production of milk fat (23% decrease) was maintained over the 20 week treatment period, whereas yields of milk and other milk components, maintenance of pregnancy and cow well-being were unaffected. A consistent reduction in the level of milk fat has also been observed in subsequent studies using calcium salts of CLA over treatment periods ranging from 3 to 20 weeks involving primiparious and multiparious cows at different stages of lactation and under different dietary and management practices (Giesy *et al*., 2002;

Bernal-Santos *et al*., 2003; Moore *et al*., 2004; Piperova *et al*., 2004; Selberg *et al*., 2004).

The preparation of dietary supplements containing CLA using other methods of rumen-protection has been investigated less extensively compared to calcium salts of CLA, but have included formulations where the protection was by treatment with formaldehyde, the formation of amide bonds and lipid-encapsulation (de Veth *et al*., 2003; Perfield *et al*., 2004b). The transfer of *trans*-10, *cis*-12 CLA to milk fat offers a convenient method to evaluate the effectiveness of rumen protection methods. While all methods resulted in a reduction in the production of milk fat, transfer efficiency of *trans*-10, *cis*-12 CLA from rumen-protected supplements was much lower than the ~22% transfer efficiency reported for investigations involving abomasal infusions (de Veth *et al*., 2004). Thus, the CLA in these formulations is protected only partially from rumen biohydrogenation and there is some indication that the rumen metabolism of a portion of the dietary supplement of CLA may result in the production of other fatty acids that are also able to inhibit milk fat synthesis (Perfield *et al*., 2002; Piperova *et al*., 2004). Additional aspects of the potential application of dietary supplements of *trans*-10, *cis*-12 CLA as a management tool are discussed by Griinari and Bauman (2003).

3.6.2. Rumenic Acid and Human Health

3.6.2.1. Cancer

Since the original discovers of the antimutagen properties of CLA (Pariza *et al*., 1979; Ha *et al*., 1987), its anticarcinogenic effects have received widespread interest. There are biomedical models for most types of cancer and many of these have been used to investigate the role of CLA as an anticarcinogen (see reviews by Scimeca, 1999; Belury, 2002; Banni *et al*., 2003; Parodi, 2004). These include the use of human cancer cell lines, transplanted cell lines and *in situ* organ site carcinogenesis models. The latter are of particular value in cancer investigations and dietary supplements of CLA have been shown to be effective in inhibiting chemically-induced skin papillomas, fore-stomach neoplasia, and preneoplastic lesions and tumors in the colon and mammary gland (see Parodi, 2004; Bauman *et al*., 2005). The majority of studies have used a mixture of CLA isomers produced synthetically from vegetable oil, typically containing 2 or 4 predominant isomers; the 2-isomer mix contains almost equal proportions of RA and *trans*-10, *cis*-12 CLA whereas the 4-isomer mixture also includes the *trans*-8, *cis*-10 and *cis*-11, *trans*-13 isomers. The anticarcinogenic effect of CLA is particularly impressive in studies on chemically-induced mammary cancer; dietary intake of CLA gives a dose-dependent reduction in the incidence and number of tumors (Ip *et al*., 1991) and is independent of the type or level of fat in the

diet (Ip *et al*., 1991, 1994). Most impressive is the fact that feeding CLA during the peripubertal period provided protection against mammary tumor development even when the carcinogen was administered at a later time (Thompson *et al*., 1997). Conversely, when rats received no CLA supplementation until they were older and had mature mammary glands, the protective effect was achieved only when CLA was fed continuously during the tumor promotion period following administration of the carcinogen (Ip *et al*., 1995).

The use of a functional food approach would have many advantages as a strategy to prevent cancer. Since CLA is found predominately in dairy fats in human diets, a series of studies have used the rat prepubertal mammary cancer model to investigate the anticarcinogenic potential of CLA when supplied as a naturally-enriched butter that was produced using dietary regimes described in Section 3.5. As discussed in Section 3.4, the majority of the RA in milk fat is synthesized endogenously from VA, and, as a consequence, the levels of VA and CLA in milk fat generally approximate a 3:1 ratio and change in concert (Bauman *et al*., 2003; Palmquist *et al*., 2005). Thus, the enriched butter is higher in both RA and VA. The initial investigation established that RA was an effective anticarcinogen when it was supplied as a dietary food in a natural form (esterified in triglycerides; Table 3.3; Ip *et al*., 1999). Importantly, tissue concentrations of RA were greater in rats fed the VA/RA-enriched butter than for rats fed a comparable amount of chemically-synthesized RA, suggesting the possibility of endogenous synthesis from VA. As discussed in Section 3.4.4, mammals possess a Δ^9-desaturase, and the ability to convert VA to RA has been demonstrated for several species, including humans (Turpeinen *et al*., 2002; see also review by Palmquist *et al*., 2005). In addition, Banni *et al*. (2001) observed that feeding rats increasing amounts of pure VA resulted in a progressive increase in tissue concentration of RA, and a corresponding reduction in the number of premalignant mammary lesions, an early marker for mammary tumors. Subsequent investigations established that dietary VA derived from VA/RA-enriched butter also resulted in a dose-dependent increase in the accumulation of CLA in the mammary fat pad, which was accompanied by a parallel decrease in tumor incidence and tumor number (Corl *et al*., 2003), and that the anticarcinogenic effects of VA were predominately, perhaps exclusively, mediated through its conversion to RA *via* Δ^9-desaturase (Lock *et al*., 2004). Therefore, VA and RA derived from milk fat are both anticarcinogenic and this series of pre-clinical investigations clearly demonstrate the feasibility of a functional food approach using dairy products enriched in VA and RA in the prevention of mammary cancer.

Premalignant lesions and tumors grow when the rate of cell proliferation exceeds cell death, and investigations to date suggest that the

Table 3.3. Bioassay of mammary cancer prevention in rats fed different sources of conjugated linoleic acids*. Adapted from Ip *et al.* (1999)

Dietary treatment	Total CLA in diet (%)	CLA content (μg/mg lipid)		Mammary tumors	
		Plasma	Mammary fat	Incidence	Total No.
Control butter	0.1	5.4[a]	7.2[a]	28/30[a] (93%)	92[a]
High CLA butter	0.8	23.3[c]	36.5[c]	15/30[b] (50%)	43[b]
Control butter & synthetic CLA	0.8	18.4[c]	26.2[b]	16/30[b] (53%)	46[b]

* Dietary treatments were initiated at weaning and continued for 30 days. All animals were then injected with methylnitrosurea (MNU) to induce mammary tumors and switched to a 5% corn oil diet with no CLA. They remained on this diet for 24 weeks and were then sacrificed for tissue analysis. Values with unlike superscripts in the same column (a, b, c) differ ($P < 0.05$).

anticarcinogenic effects of CLA involve a multitude of mechanisms. These include a decrease in cell proliferation, an increased rate of apoptosis, inhibition of angiogenesis, modulation of the immune cell environment, alteration in the eicosanoid signalling pathways and a possible antioxidant role (see Belury, 2002; Banni *et al.*, 2003; Ip *et al.*, 2003). Particular mechanisms may vary in importance depending on the tissue-specific process being regulated, and the opportunity to exploit the diversity in the mechanism of action of CLA may form the basis for the range in tissues and cancer types in which CLA is effective.

Evaluating the specific role of CLA in health maintenance and the prevention of cancer in humans is difficult. Since cancer takes many years to develop, documenting that dietary CLA is beneficial in health maintenance and the prevention of this disease is a major challenge. Results, from epidemiological studies gave conflicting results (Aro *et al.*, 2000; Voorrips *et al.*, 2002; Chajes *et al.*, 2003; McCann *et al.*, 2004). This inconsistency is not surprising. Dairy products are used in recipes for many manufactured food products, and estimating CLA intake is further complicated by the fact that CLA is a fatty acid and dairy products vary widely in fat content, milk fat varies widely in CLA content, and analysis of RA is difficult and reported values are often inaccurate (see reviews by Parodi, 2004; Bauman *et al.*, 2005). Another approach would be dietary interventions using biomarkers as end points to predict reduced cancer risk, but to date there are no consensus biomarkers for breast cancer and many other cancer types. Clearly, assessing the role of dietary CLA in functional foods for the prevention of cancer presents some unusual difficulties, and thus many of the traditional approaches to evaluate human health effects have substantial limitations.

3.6.2.2. Atherosclerosis

Investigations of the effects of CLA on atherosclerosis are limited compared with anticarcinogenic studies. A number of animal studies have demonstrated that dietary supplementation with mixtures of CLA isomers can reduce the development of atherosclerotic lesions (Lee *et al.*, 1994; Nicolosi *et al.*, 1997; Kritchevsky *et al.*, 2000; 2002; Wilson *et al.*, 2000) and even induce the regression of pre-existing lesions in rabbits (Kritchevsky *et al.*, 2000; 2004). However, one study, in the atherosclerosis-susceptible C57BL/6 mouse, showed that CLA had no effect on atherosclerotic lesions, and could even promote their development (Munday *et al.*, 1999). Studies with pure isomers recently demonstrated that RA and *trans*-10, *cis*-12 CLA were equally effective in reducing cholesterol-induced atherogenesis in rabbits (Kritchevsky *et al.*, 2004). Of particular significance is that RA induced the regression of atherosclerotic lesions in the $ApoE^{-/-}$ knockout mouse (Toomey *et al.*, 2003). This model has been used widely in studies of atherosclerosis because it spontaneously develops lesions on a regular low-fat, low-cholesterol diet with a histopathology similar to lesions that develop in humans (Meir and Leitersdorf, 2004).

Changes in both total plasma cholesterol and individual lipoprotein cholesterol concentrations have been implicated as major determinants of the risk of atherosclerosis and this has led to a number of studies which specifically investigated the effects of CLA on cholesterol and lipoprotein metabolism in animal models. Most have used a synthetic source composed of a mixture of CLA isomers, and results have been inconsistent with some showing beneficial changes in blood lipid variables while others have shown no effect (see Bauman *et al.*, 2005). We recently completed a study using the Golden Syrian hamster to examine the potential of CLA when fed as a component of a functional food (VA/RA-enriched butter) as part of a diet that was high in cholesterol (0.2%) and fat (20%). Compared with the control animals, those fed the VA/RA-enriched butter showed a number of beneficial effects, including reduced total plasma cholesterol and VLDL and LDL cholesterol lipoproteins, suggesting that CLA may modify the production of atherogenic lipoproteins by the liver (Lock *et al.*, 2005b). In addition, the VA/RA-enriched butter produced a less atherogenic profile than an equivalent diet in which the VA/RA-enriched butter was replaced by *trans* fatty acids from partially hydrogenated vegetable oil (Lock *et al.*, 2005b). Consistent with these findings, it has been proposed (McLeod *et al.*, 2004) that RA could, in the absence of other CLA isomers, improve hepatic lipid metabolism. This may explain why VA/CLA-enriched butter elicited such impressive effects compared to studies in which synthetic CLA isomer mixtures are used, since naturally-derived sources of CLA provide essentially only RA.

It is important to note that an elevated and/or altered plasma lipid level is only one of a wide range of risk factors that contribute to the clinical manifestations of cardiovascular disease in humans (Lusis, 2000). Consequently, in some studies, the reduced incidence of atherosclerosis in animals fed CLA was not accompanied by an improvement in the plasma lipid profile during the CLA feeding phase (Wilson *et al*., 2000). Reasons for these effects are not understood fully. However, atherosclerosis can also be considered as a chronic inflammatory disease (Libby, 2002) and several important anti-inflammatory effects have been associated with the use of RA; these include a reduction in the expression of COX-2, PGE_2, reduced release of nitric oxide, a decreased production of pro-inflammatory cytokines, and PPARγ activation (Urquhart *et al*., 2002; Yu *et al*., 2002; Toomey *et al*., 2003).

Since results from studies with biomedical models indicate potential, there is of obvious interest in the effects of RA consumption in foods on the risk of atherogenesis in humans. The use of surrogate biomarkers for disease risk is more readily achievable for atherosclerosis than for cancer in humans and a number of genetic and environmental risk factors have been identified, with the relative abundance of the different lipoproteins being of primary importance (Lusis, 2000). To date, there have been no epidemiological studies that have examined the intake of CLA derived from foods with the risk of atherosclerosis. However, as discussed in Section 3.6.2.1, the challenge of adequately evaluating the effect of dietary intake of CLA from different food sources presents some special limitations.

Several human intervention studies involving dietary supplements of CLA in the form of capsules have shown plasma lipid variables as secondary observations, but most utilized mixed isomers of CLA and gave variable results (see Bauman *et al*., 2005). However, two recent studies examined the specific effects of RA on blood lipids in healthy subjects; a CLA supplement containing a 50:50 mixture of RA and *trans*-10, *cis*-12 CLA significantly improved plasma triacylglycerol and VLDL metabolism, with an 80:20 CLA isomer blend (RA:*trans*-10, *cis*-12 CLA) significantly reducing the concentration of VLDL-cholesterol, providing further evidence for the role of RA in altering hepatic lipid metabolism (Noone *et al*., 2002). Utilizing pure CLA isomers, it was observed that RA and *trans*-10, *cis*-12 CLA had opposing effects on blood lipids in healthy humans; plasma triacylglycerol, total plasma cholesterol, LDL-cholesterol and LDL:HDL-cholesterol were all lower during supplementation with RA compared to *trans*-10, *cis*-12 CLA (Tricon *et al*., 2004). A companion study showed that CLA isomers were readily incorporated into plasma and cellular lipids to a similar extent and in a dose-dependent manner (Burdge *et al*., 2004). Although these data are limited, they provide support that some of the anti-atherosclerosis effects

of CLA reported in animal models will extend to humans. As previously mentioned, often in atherosclerosis studies utlizing animal models, the beneficial effects did not involve alterations in plasma lipids. Thus, CLA studies in humans that focus on the possible role of eicosanoid- and cytokine-related effects could also be of importance.

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