# BIOSYNTHESIS OF CONJUGATED LINOLEIC ACID IN RUMINANTS AND HUMANS

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# I. INTRODUCTION

## A. BACKGROUND

Nutritional quality is becoming a major issue in food choices because of growing consumer awareness of the link between diet and health. As a consequence, there is increasing consumer acceptability of the concept of "functional foods," a generic term used to describe foods or food components that have beneficial effects on human health above that expected on the basis of nutritive value (Milner, 1999). In other words, functional foods must have a relevant effect on well-being and health or cause a reduction of disease risk (Roberfroid, 1999). One of these functional food components is conjugated linoleic acid, a fatty acid found in milk fat and ruminant meat. The term *conjugated linoleic acid* (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. Numerous isomers of CLA have been identified in food products, and these differ by position (e.g., 7–9, 8–10, 9–11, 10–12) or geometric orientation (cis-trans, trans-cis, cis-cis, and trans-trans) of the double-bond pair. The predominant source of CLA in human diets is ruminant-derived food products. In the United States, dairy products provide about 70% of the intake, and beef products account for another 25% (Ritzenthaler *et al.*, 2001) (Figure 1), and similar values for the contribution of different food classes are found for other countries (Parodi, 2003). The presence of CLA in ruminant milk has been known for more than 60 years. Scientists at the University of Reading, United Kingdom, first demonstrated that fatty acids obtained from summer butter differed from those obtained from winter butter by exhibiting a much stronger spectrophotometric adsorption at 230 μm (Booth *et al.*, 1933). Subsequently, Moore (1939) concluded that the adsorption at 230 μm was due to two conjugated double bonds. Parodi (1977) first identified *cis*-9, *trans*-11 octadecadienoic acid as the predominant CLA isomer in milk fat, and its structure is compared with linoleic acid in Figure 2. Although many isomers of CLA occur in ruminant fat, *cis*-9, *trans*-11 CLA accounts for about 75–90% of the total. The second most common isomer is *trans*-7, *cis*-9 CLA, representing about 10% of the total. *Trans*-7, *cis*-9 CLA co-elutes with the *cis*-9, *trans*-11 isomer on most gas liquid chromatograms, and identity of this isomer went unrecognized until it was isolated by Yurawecz *et al.* (1998) using combinations of silver nitrate high-performance liquid chromatography (HPLC), gas liquid chromatography, mass spectrometry, and Fourier transform infrared spectroscopy. The remainder of the CLA is composed of other trans-trans, trans-cis, cis-trans, or cis-cis forms, with each isomer typically representing a small portion (<1%) of the total (Bauman *et al.*, 2003; Parodi, 2003) (Table I). The

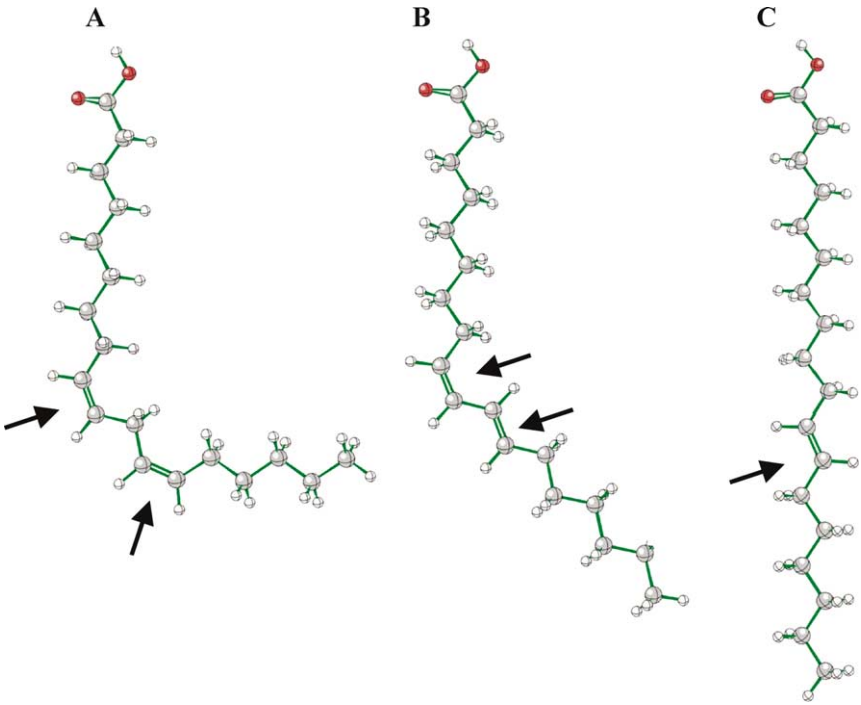


FIG. 1 Chemical structures of linoleic acid (*cis*-9, *cis*-12 18:2) (A), *cis*-9, *trans*-11 CLA (B), and *trans*-11 18:1 (C). Arrows indicate location of double bonds. (Adapted from Bauman *et al.*, 2004.)

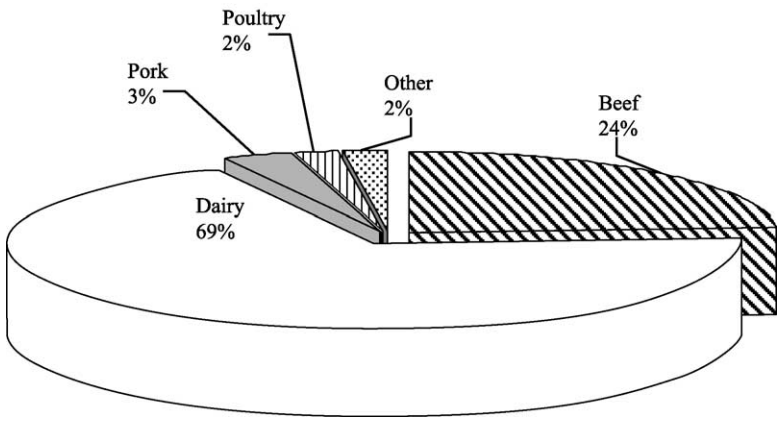


FIG. 2 Distribution of CLA sources in the U.S. diet. (Calculated from data by Ritzenthaler *et al.*, 2001.)

TABLE I
BENEFICIAL HEALTH EFFECTS OF CLA REPORTED FROM BIOMEDICAL STUDIES WITH ANIMAL MODELS

| Biological effect |
|---|
| Anticarcinogenic (*in vivo* and *in vitro* studies) |
| Antiatherogenic |
| Altered nutrient partitioning and lipid metabolism |
| Antidiabetic (type II diabetes) |
| Immunity enhancement |
| Improved bone mineralization |

trivial name "rumenic acid" (RA) has been proposed for *cis*-9, *trans*-11 CLA (Kramer *et al.*, 1998) and is used when discussing this isomer. A summary of all known fatty acids in milk was published by Jensen (2002).

The plethora of research related to CLA over the past 20 years was prompted by the identification of CLA as an anti-mutagen present in cooked beef (Ha *et al.*, 1987; Pariza *et al.*, 1979). Since these original findings, a number of potential health benefits of CLA have been reported (Table I). Health effects of CLA were established initially in biomedical studies of cancer, and subsequent investigations with animal models and *in vitro* cell cultures demonstrated that CLA was anticarcinogenic for many types of cancer (Belury, 2002; Parodi, 2002; Whigham *et al.*, 2000). Both RA and *trans*-10, *cis*-12 CLA are effective in reducing the formation of premalignant lesions in the rat mammary gland 6 weeks after carcinogen administration (Ip *et al.*, 2002). This is the reason there is enormous interest in the "functional food" properties of food products containing RA. Particularly noteworthy is that RA is a potent anticarcinogen when supplied in a natural form (esterified triglyceride) as a natural food component. Dietary consumption of RA-enriched butter was effective in reducing the incidence of tumors in a rat model of mammary carcinogenesis (Ip *et al.*, 1999). Though not as extensively investigated, other studies have demonstrated beneficial effects of CLA on atherosclerosis and related variables in a number of animal models (Kritchevsky, 2003). Using pure isomers, RA and *trans*-10, *cis*-12 CLA were shown to be equally effective in reducing cholesterol-induced atherogenesis in rabbits (Kritchevsky, 2003). Further, CLA has other beneficial health effects in studies with animal models. These include reducing the onset and severity of diabetes and obesity, immune modulation, and altering the rate of bone formation (Table I). Although research in these areas is limited compared with the effects of CLA on cancer, they merit consideration and further research. For in-depth reviews of these, see Pariza (1999), Whigham *et al.* (2000), Belury (2002), and Parodi (2002).

Because the presence of CLA in the human diet is reliant on ruminant products, this chapter first addresses the synthesis of CLA in ruminants. The presence of CLA in ruminant milk and meat is related to rumen fermentation and its synthesis by microorganisms through the process of biohydrogenation (BH) of dietary unsaturated fatty acids. Thus, the effect of diet and processes within the rumen is reviewed. The role of endogenous synthesis of CLA in mammalian tissues has been discovered, and this will be discussed also first as it contributes to the occurrence of CLA in ruminant products and second the significance of endogenous synthesis as a source of CLA in humans and other species.

## B. THE RUMINANT DIET

Ruminant diets are extremely varied, depending on species and productive function. Diets range from all forage of variable quality (pasture, corn silage, grass or legume hay, or mixed grass and legume hay, and hay silage) to combinations of forage, cereals, and protein supplements. Many byproducts of the food industry, also highly variable in quality, may be included. A general characteristic is that ruminant diets are high in fiber (generally >30% cell wall constituents) and low in lipid content. Fatty acid content of grass and legume forages is generally lower than 5% of the dry matter and consists of a high proportion (>50%) of α-linolenic acid (all *cis* 18:3n-3). Corn silage and cereal grains contain similar quantities of fatty acid, of which linoleic acid (*cis, cis* 18:2 n-6) predominates. Diets may contain up to 5% of dry matter from supplemental fats, varying from highly saturated fats such as tallow, oilseeds, such as canola, cottonseed, and soybeans, and byproducts of vegetable oil processing. A detailed description of dietary fatty acids in ruminant diets is provided by Viviani (1970), and in more condensed form, by Palmquist (1988) and Harfoot and Hazlewood (1997); the latter also includes a modern summary of fatty acids found in ruminal organisms and their metabolic end products. Even though ruminant diets contain predominantly unsaturated fatty acids, ruminant meat and milk contain much higher levels of saturated fatty acids due to extensive BH of dietary unsaturated fatty acids in the rumen.

## C. THE RUMEN MICROBES

The general consensus from numerous *in vitro* studies is that rumen bacteria are primarily responsible for BH (Harfoot and Hazlewood, 1997). Even though the rumen contains up to $10^{11}$ viable cells/ml and a diverse range of bacteria (Hungate, 1966), relatively few bacterial species capable of BH

have been identified, and the relative importance of individual strains to ruminal lipid metabolism *in vivo* remains largely unclear. Of those species shown to be capable of BH, *Butyrivibrio fibrisolvens* is the most extensively studied, but the metabolic activity of other species including *Ruminococcus, Eubacterium,* and *Fusocillus* strains also have been characterized (Harfoot and Hazlewood, 1997). The role of BH in ruminal metabolism has been a subject of debate, and early studies promoted the suggestion that the biological importance of BH was associated with providing fatty acid substrates for incorporation into bacterial membranes or was an essential mechanism for removing reducing equivalents (Harfoot and Hazlewood, 1997). However, most of the experiments to date are consistent with the view that the principle role of BH is to reduce the toxic effects of unsaturated fatty acids on bacterial growth (Kemp and Lander, 1984; Kemp *et al.*, 1984b). The dynamics of bacterial growth, substrate supply, and ruminal turnover were described in detail by Hungate (Chapter V, 1966) and succinctly summarized by Viviani (1970) as follows: "At an outflow of rumen contents of 6–8% $hr^{-1}$ the mean generation time must be 12 hours if organism numbers are to be maintained. However, many are able to divide two or three times per hour; therefore, the rate of growth is far less than maximal, causing them to be in a maintenance, rather than a growing state. Thus, linolenic acid was BH to octadecanoic acid when incubated with *B. fibrisolvens* for 21 hours, whereas little BH occurred when incubation was limited to 2 hours." Significance of these observations is seen in the later work of Kim *et al.* (2000) (see Section II.C).

There is little evidence indicating that rumen protozoa are capable or involved in BH (Harfoot and Hazlewood, 1997; Williams and Coleman, 1988), and it is arguable that protozoa could satisfy their lipid requirements through the ingestion of rumen bacteria, chloroplasts, and other plant lipids.

## II. RUMINAL SYNTHESIS OF CLA

### A. LIPOLYSIS

Before BH of fatty acids can take place, plant lipids must become free of surrounding matrix by mastication and microbial digestive processes, followed by lipolysis of ester linkages. The lipolytic step is believed to be rate limiting for BH (Harfoot and Hazlewood, 1997). Dawson and Hemington (1974) described the digestion of grass lipids and pigments. Monogalactosyl and digalactosyl diacylglycerides are released rapidly as chloroplasts are ruptured by mastication. Deacylation was linear and more rapid for monoacylglycerides than diacylglycerides in the rumen content. Products were

fatty acids and monogalactosyl and digalactosyl glycerol; no free acylglycerol was found; conversely, monoacylglycerol and diacylglycerol were found as intermediates of lipolysis when triacylglycerol (as peanut oil) was added to the medium (Clarke and Hawke, 1970). The latter authors reported that fatty acid was released linearly in strained rumen fluid up to concentrations of 1 mg of triacylglycerol/ml. Using much higher substrate concentrations (20–100 mg/ml), Beam *et al.* (2000) also reported that rate of lipolysis *in vitro* decreased at high substrate concentrations, with lag times before initiation of lipolysis ranging from 1.3 to 2.5 hours. Using $^{14}$C-labeled substrate at 0.5 mg/ml, Hawke and Silcock (1970) observed that lipolysis in strained rumen fluid conforms to first-order kinetics, with a rate constant of 0.0267 $min^{-1}$ and a 32-minute lag. They concluded "that under normal conditions lipolysis of ingested lipids would be sufficiently rapid in the rumen to allow the full biohydrogenating capacity of the rumen microorganisms to be realized." However, differences in BH products of esterified and nonesterified linoleic acid have been shown (Moore *et al.*, 1969; Noble *et al.*, 1974). Both *in vitro* and *in vivo* presentation of free linoleic acid resulted in accumulation of vaccenic acid (VA), whereas stearic acid was the main product after infusion of corn oil (see later discussion). These observations suggest that the rate of lipolysis limits the availability of free linoleic acid in the rumen, resulting in complete BH when unsaturated fatty acids are provided in the glyceride form. However, an excessive supply of unsaturated oil in ruminant diets has negative effects on ruminal metabolism and BH (Jenkins, 1993). Several factors of the ruminal environment modify the rates of ruminal lipolysis and BH (Gerson *et al.*, 1983, 1985, 1986). These studies show that increasing dietary contents of nitrogen and fiber increased the rate of lipolysis and that lipolytic rate was decreased with increases in forage maturity. Low ruminal pH levels decrease lipolysis (Latham *et al.*, 1972); very little lipolysis occurs at pH levels of less than 6.0, whereas BH was inhibited only partially at a pH level of 5.2 (van Nevel and Demeyer, 1996).

The rate of lipolysis is related to melting point; seed oils (linseed, soya) > palm oil = tallow. Fish oils are hydrolyzed more slowly, probably caused by steric hindrance of the ester bonds (Miller and Cramer, 1969; Palmquist and Kinsey, 1994). Hydrogenated tallow (iodine number < 30) lipolysis rate was slow (Palmquist and Kinsey, 1994) or absent (Beam *et al.*, 2000), likely caused by insolubility of the substrate.

## B. ISOMERIZATION

Biosynthesis of CLA by rumen bacteria arises from isomerization of polyunsaturated dietary C18 fatty acids; CLA and other ethenoic isomers occur as intermediates in the pathways of ruminal BH, whereby the unsaturated

dietary fatty acids are metabolized to stearic acid as the primary end product (Harfoot and Hazlewood, 1997). The process of BH is associated with the activity of bacteria adhering to rumen particulate matter (Gerson *et al.*, 1988; Harfoot *et al.*, 1973). Conditions required for BH to take place were described by Kepler *et al.* (1970); namely a free carboxyl group, an all-*cis* Δ-9 pentadiene system and a chain length of 18 C atoms. All methylene-interrupted *cis, cis* octadecadienoic fatty acids were examined as substrates for BH by Garcia *et al.* (1976) and by Kemp *et al.* (1984b). Although BH of all the unsaturated fatty acids occurred, conjugation of the double bonds before BH was required only for fatty acids with pentadiene structure Δ-2, 5 and Δ-9,12. Kepler and Tove (1967) purified and characterized the membrane-bound enzyme linoleate Δ-12 *cis*, Δ-11 *trans* isomerase, which catalyzes the first step in the BH of linoleic acid, from *B. fibrisolvens.* The product of this enzyme is Δ-9 *cis*, Δ-11 *trans*-octadecadienoic acid (RA), the predominant CLA isomer found in ruminant foods.

Other CLA isomers have been identified in lesser amounts in ruminant foods. Amounts and relative proportions of individual CLA isomers may be altered in response to changes in the animal's diet; for example, the *trans*-10, *cis*-12 isomer is increased by inclusion of unsaturated fatty acids in the diet and a low ruminal pH (Bauman and Griinari, 2001; Piperova *et al.*, 2002). *Trans*-10, *cis*-12 CLA is quantitatively a minor BH intermediate (Duckett *et al.*, 2002; Lock and Garnsworthy, 2002; Shingfield *et al.*, 2003) but is of major interest because of its physiological effects on fat metabolism (Baumgard *et al.*, 2000; Peterson *et al.*, 2003a). The origin of *trans*-10, *cis*-12 18:2 was unknown until a report by Kim *et al.* (2002) showed its synthesis by *Megasphaera elsdenii* strain YJ-4. *M. elsdenii* is a lactate fermenter that thrives in the rumen of animals fed high-grain diets, conditions that are known to be favorable for low milk fat syndrome in cattle (Bauman and Griinari, 2003). The characteristics of the enzyme are similar to those described for linoleate Δ-12 *cis*, Δ-11 *trans* isomerase (Kim *et al.*, 2000) in *B. fibrisolvens,* the exception being that *trans*-10, *cis*-12 CLA is the product, rather than RA. *M. elsdenii* strain YJ-4 was not found in the rumen of all animals fed high-grain diets; indeed, other strains of *M. elsdenii* often predominated, explaining the phenomenon that not all cattle are susceptible to induction of the low milk fat syndrome. In addition to the likelihood of low amounts synthesized, *trans*-10, *cis*-12 CLA is a substrate for the reductase of *B. fibrisolvens* and was BH to *trans*-10 18:1 at one-third the rate of conversion of RA to *trans*-11 18:1 (Kepler *et al.*, 1966).

Ruminal synthesis of *trans, trans* CLA isomers with double bonds in positions 9, 11 and 10, 12 also are enhanced when diets contain high amounts of concentrates (Piperova *et al.*, 2002) or are supplemented with fish oil (Shingfield *et al.*, 2003). Formation of *trans, trans* CLA is unexpected

based on established BH pathways (Harfoot and Hazlewood, 1997). The identification of *trans, trans* CLA isomers in omasal (Shingfield *et al.*, 2003) or duodenal (Piperova *et al.*, 2002) digesta of dairy cows raise some intriguing questions with regard to CLA synthesis in the rumen. Possibly linoleate Δ-12 *cis*, Δ-11 *trans* isomerase is less specific than previously thought, with ability to catalyze the formation of *trans*-9, *trans*-11 and *trans*-10, *trans*-12 CLA from linoleic acid. An alternative explanation would be that rumen bacteria express several linoleate isomerases that have not been identified and characterized.

Characterizing metabolic pathways of BH in ruminal microorganisms is extremely difficult, for several reasons. First, the organisms are strict anaerobes, which require extreme methodologies for their culture. Second, as is discussed later in this chapter, complete BH occurs in very few organisms capable of BH, thus requiring multiple species to carry out the process. Third, very few of the isolates that have been screened have BH capability; for example, Kemp *et al.* (1975) screened more than 200 isolates, 30 showed limited BH activity, and of these, only five had sufficient activity to warrant further work. Finally, many of the original well-characterized BH strains have been lost (van de Vossenburg and Joblin, 2003), thereby preventing further pursuit of their characteristics.

## C. THE BIOHYDROGENATION PROCESS

Numerous *in vitro* and *in vivo* studies have elucidated the major pathways of ruminal BH (Figures 3 and 4) (Harfoot and Hazlewood, 1997). Of significance for this discussion is that the bacteria (Group A) responsible for isomerization to the conjugated diene and hydrogenation of the *cis*-Δ-9 bond are thought to be unable to complete the BH of the *trans*-11 18:1 intermediate. The reduction of *trans*- and *cis*-octadecenoic acids is thought to occur in separate organisms, collectively known as the group B bacteria (Harfoot and Hazlewood, 1997). Thus, complete BH of unsaturated fatty acids requires a balance between the two groups A and B. Reduction of *trans*-octadecenoic acids to stearic acid is believed to be the rate-limiting step, so these fatty acids can accumulate in the rumen (Keeney, 1970). However, rates of BH are higher for *trans*-octadecenoic acids with double bonds in positions Δ8–Δ10 than for those with bonds at Δ5–Δ7 or Δ11–Δ13 (Kemp *et al.*, 1984a).

The reduction of RA to VA is catalyzed by *cis*-9, *trans*-11 octadecadienoic acid reductase. Isolation of this membrane-bound enzyme from *B. fibrisolvens* indicates that it has an absolute requirement for iron and expresses maximal activity at a pH between 7.2 and 8.2 (Hughes *et al.*, 1982). Pure culture studies with *B. fibrisolvens* also have shown that the reductase is not

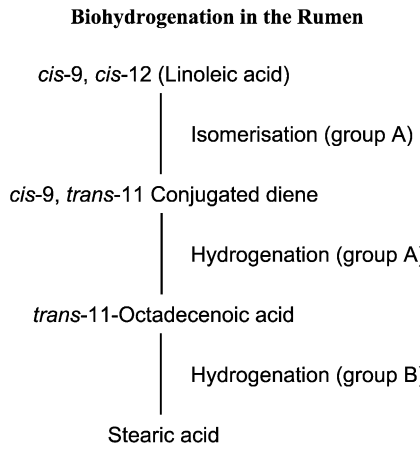


FIG. 3 Scheme for the biohydrogenation of linoleic acid; group A and group B refer to the two classes of biohydrogenating bacteria. (Used, with permission, from Harfoot and Hazlewood, 1997.)

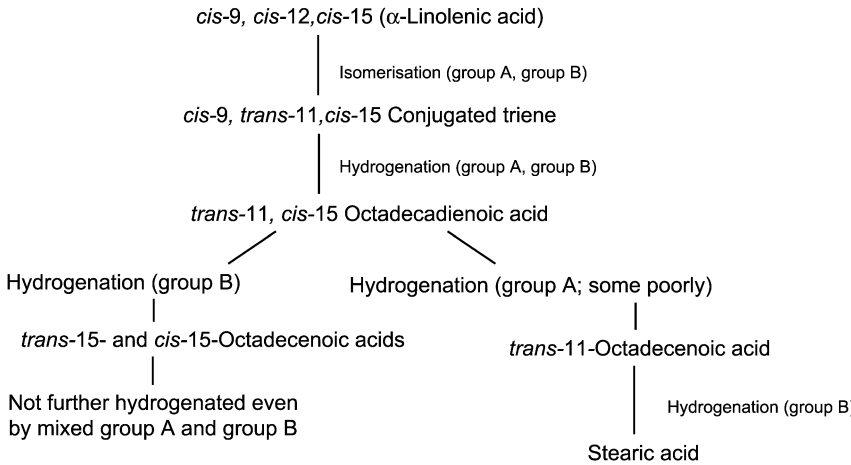


FIG. 4 Scheme for the biohydrogenation of α-linolenic acid; group A and group B refer to the two classes of biohydrogenation bacteria. (Used, with permission, from Harfoot and Hazlewood, 1997.)

highly specific and can convert *trans*-10, *cis*-12 CLA to *trans*-10 18:1 (Kepler *et al.*, 1966).

It has long been known (Noble *et al.*, 1969, 1974; Polan *et al.*, 1964) that an excess amount of free linoleic acid inhibits the final BH of VA to stearic

acid. Thus, excess free linoleic acid in the rumen environment leads to accumulation of VA. Research has shown that the highly unsaturated long-chain fatty acids of fish oil are even more effective in causing the accumulation of VA and other trans monoenoic acids (Shingfield *et al.*, 2003). These studies confirm the report of Kemp and Lander (1984), showing that stearic acid was the predominant end-product when cultures of groups A and B in late exponential growth phase were combined, but VA accumulated when small amounts of inocula were used; it was presumed that group A organisms overgrew those of group B. Presumably, group B bacteria are more sensitive to the toxic effects of long-chain polyunsaturated fatty acids (PUFAs) than group A bacteria, and therefore, VA and other trans monoenes accumulate when PUFA concentrations are increased.

Kim *et al.* (2000) brought new insight to the effects of linoleic acid concentration on RA synthesis by *B. fibrisolvens.* Growing cultures tolerated only low concentrations of linoleic acid; these cultures did not produce significant amounts of CLA until linoleic acid concentration was high enough to inhibit BH (reduction of RA to VA), upon which growth was inhibited and cell lysis commenced. Isomerization of linoleic acid was very rapid, but the isomerase did not recycle as do most enzymes in order to catalyze more substrate; CLA accumulation was proportional to cell concentration. The isomerase and reductase enzymes were linked and free CLA was not released as an intermediate. Because CLA was found to be as toxic as linoleic acid, there was no survival advantage for the organisms to release CLA. The authors concluded that CLA found in the medium (ruminal or intestinal contents) may be due to linoleic acid–dependent bacterial inactivation, cell death, or lysis. Under conditions of less than toxic amounts of linoleic acid (or other inhibitory PUFAs), the product of BH is released as VA. The organism was found to tolerate higher concentrations of linoleic acid after adaption; this could explain the common observation of high initial concentrations of CLA in milk when unsaturated fats are fed, decreasing as time on diet increases (Bauman *et al.*, 2000).

The initial step in BH of α-linolenic acid is similar to that for linoleic acid, resulting in formation of a conjugated diene, with an additional isolated double bond (Figure 4). However, some isolates of group B bacteria, as well as group A bacteria, are able to isomerize linolenic acid (Harfoot and Hazlewood, 1997). The subsequent hydrogenation of the *cis*-Δ-9 bond yields *trans*-11, *cis*-15 18:2. Further hydrogenation by group A organisms yields VA, whereas hydrogenation by group B organisms results in the unique products of either *trans*-15 or *cis*-15 monoenes. The latter are not hydrogenated further and thus are true end-products (Harfoot and Hazlewood, 1997).

Other studies have shown that a strain of *Butyrivibrio hungatei* isolated from a cow grazing ryegrass-clover swards is capable of metabolizing both

linoleic and linolenic acids to stearic acid (van de Vossenberg and Joblin, 2003). Even though this bacterium produced transient intermediates according to established pathways, it is clear that in some cases complete BH of PUFAs may not necessarily require the involvement of two groups of complementary bacteria, as was previously thought (Kemp and Lander, 1984).

In addition to linoleic and α-linolenic acids, the BH of γ-linolenic acid (all *cis* 6, 9, 12 18:3) has been investigated using pure strains of *Butyrivibrio* S2 and *Fusocillus babrahamensis in vitro* (Kemp and Lander, 1983). The group B bacterium (*Fusocillus*) was able to metabolize γ-linolenic acid completely to stearic acid, whereas the group A bacterium (*Butyrivibrio*) produced *cis*-6, *trans*-11 octadecadienoic acid. It has been suggested that the BH of γ-linolenic acid is analogous to that of α-linolenic acid and proceeds via isomerization to a conjugate (*cis*-6, *cis*-9, *trans*-11 18:3) that is sequentially reduced to *cis*-6, *trans*-11 18:2, vaccenic acid, and stearic acid (Figure 5). Even though most conventional ruminant feeds do not contain γ-linolenic acid, this fatty acid is present in certain oilseeds including evening primrose and borage, but the effects of these lipids on ruminal BH *in vivo* or CLA concentrations in milk and meat have not been investigated.

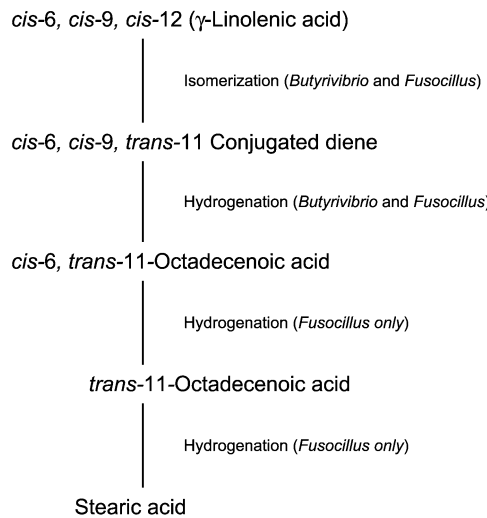


FIG. 5 Scheme for the biohydrogenation of γ-linolenic acid; the *Butyrivibrio* used in the study was a group A biohydrogenator, and the *Fusocillus* was a group B biohydrogenator (Kemp and Lander, 1983). Compare Figure 4 for biohydrogenation of α-linolenic acid. (Used, with permission, from Harfoot and Hazlewood, 1997.)

The effects of pH and amounts of linoleic and linolenic acids on extent of BH *in vitro* and proportions of BH intermediates, including monoenoic and dienoic isomers, were examined by Troegeler-Meynadier *et al.* (2003). When linoleic acid concentration was held constant, its disappearance declined when mean pH was less than 6.0, compared with a pH level of 6.5. Increasing concentration of linolenic acid decreased linoleic acid disappearance, suggesting an inhibition of isomerization. Lower pH levels decreased the ratio of *trans*-10:*trans*-11 18:1 intermediates. When linoleic acid concentration was increased, the proportion of linoleic acid disappearing declined, but the amount disappearing increased, without changing the *trans*-10:*trans*-11 ratio, suggesting a maximum capacity of isomerization rather than inhibition. Increasing initial concentrations of linoleic acid resulted in high amounts of VA and increasing stearic acid with time, suggesting a maximal capacity for the second reductive step of BH. High concentrations of linolenic acid did not affect amounts of RA and *trans*-18:1 formed or the *trans*-10:*trans*-11 ratio. Authors concluded that a ruminal pH near neutrality with high concentrations of linoleic acid should support maximal synthesis of VA and RA.

## D. RUMINAL SYNTHESIS OF CLA

Relatively few measurements of ruminal CLA synthesis *in vivo* are available. Experiments across a range of diets have been conducted with sheep (Kucuk *et al.*, 2001), beef cattle (Duckett *et al.*, 2002, Lee *et al.*, 2003; Sackmann *et al.*, 2003), non-lactating cows (Lock and Garnsworthy, 2002), and lactating dairy cows (Piperova *et al.*, 2002; Shingfield *et al.*, 2003). Most studies indicate that RA is, in most cases, the most important CLA isomer formed in the rumen, but the amounts produced are relatively small compared with *trans*-18:1 BH intermediates. Flows of RA in the duodenum of sheep have been reported to vary between 0.12 and 0.20 g/day (Kucuk *et al.*, 2001) depending on the amount of forage in the diet. Comparison of RA synthesis in steers (0.1–2.7 g/day; Duckett *et al.*, 2002; Lee *et al.*, 2003; Sackmann *et al.*, 2003), non-lactating cows (0.3–0.5 g/day; Lock and Garnsworthy, 2002), and lactating cows (0.2–1.7 g/day; Piperova *et al.*, 2002; Shingfield *et al.*, 2003) indicate that diet type rather than level of intake is the major determinant of the amounts of RA synthesized in the rumen. Even though published reports indicate limited formation in the rumen, flows of RA entering the omasal canal can be as much as 9.9 g/day in cows fed grass silage–based diets supplemented with 750 g/day of sunflower oil (Shingfield *et al.*, unpublished observations).

RA is not the only CLA isomer formed in the rumen, and analysis of digesta by gas chromatography (Duckett *et al.*, 2002; Sackmann *et al.*, 2003)

TABLE II
DISTRIBUTION AND RUMINAL OUTFLOW OF *TRANS* 18:1 AND ISOMERS OF CONJUGATED 18:2 FATTY ACIDS IN GROWING AND LACTATING CATTLE[a,b]

| 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 |

[a]Adapted from Bauman *et al.* (2003).
[b]Data derived from three studies based on omasal (Shingfield *et al.*, 2003) or duodenal sampling (Duckett *et al.*, 2002; Piperova *et al.*, 2002).

or more comprehensive determinations using silver-ion HPLC (Corl *et al.*, 2002; Piperova *et al.*, 2002; Shingfield *et al.*, 2003) have shown that a range of isomers is formed (Table II). The latter have shown that *trans*-9, *trans*-11 CLA, *trans*-11, *cis*-13 CLA, and *trans*-11, *trans*-13 CLA are the most abundant after RA, with *trans*-10, *cis*-12 CLA being only a minor component. However, duodenal flow of the *trans*-10, *cis*-12 isomer has been reported to be higher than that of RA in steers fed corn–based diets supplemented with corn oil (Duckett *et al.*, 2002) or for low-forage diets containing sunflower oil (Sackmann *et al.*, 2003).

### E. OTHER MONOENOIC ISOMERS

The established major pathways of BH describe the formation of VA but do not account for the occurrence of other 18:1 fatty acids identified in rumen digesta (Katz and Keeney, 1966) (Table II). Following detection of a large number of positional *trans* isomers of dienoic and monoenoic fatty acids, after linoleic acid was incubated with rumen contents, Ward *et al.* (1964)

suggested that BH of dienoic acid to monoenoic acids was associated with double-bond migration. However, as Griinari and Bauman (1999) noted, there is little evidence to suggest that processes of BH in the rumen are analogous to the extensive double-bond migration that occurs when vegetable oils are heated with metal catalysts. Furthermore, certain rumen bacteria isomerize *cis* monoenoic fatty acids, forming both *cis* and *trans* bonds (Kemp *et al.*, 1984a). Mosley *et al.* (2002) examined the BH of $^{13}$C oleic acid by mixed ruminal microorganisms; extensive labeling was found in *trans* monoenes with double bonds from Δ-6 to Δ-16, except Δ-8, as well as in stearic acid. A similar distribution was observed when $^{13}$C-labeled elaidic acid was the precursor (Proell *et al.*, 2002). Studies of a strain of *B. hungatei* have shown that this rumen bacterium produces a mixture of trans positional isomers during the BH of oleic acid (van de Vossenberg and Joblin, 2003). Formation of the mixed 18:1 isomers is not spontaneous but requires enzyme mediation (Mosley *et al.*, 2002); however, the enzymes and underlying mechanisms have not been identified.

### F. SUMMARY OF THE MECHANISM OF BIOHYDROGENATION

Fatty acids with the *cis*-9, *cis*-12 pentadiene system are isomerized to the *cis*-9, *trans*-11 conjugated diene before BH takes place; the only other pentadiene that requires isomerization before BH is *cis*-2, *cis*-5. Other unsaturated bonds are hydrogenated directly. The predominant BH end-product is stearic acid; however, monoenoic, conjugated diene, and isolated dienes, with both *cis* and *trans* double bonds, have been described (Kemp *et al.*, 1975; Shingfield *et al.*, 2003).

### G. FEEDING EFFECTS ON RUMINAL BH

In view of the potential benefits to human health, numerous studies have been conducted to examine nutritional strategies to enhance the CLA content of ruminant meat and milk. The diet of the ruminant animal is an important determinant of CLA content in milk and meat, and several generalizations can be made; levels of RA are higher in milk or meat from animals grazing pasture (Dhiman *et al.*, 1999; Kelly *et al.*, 1998b; Lawless *et al.*, 1998; Stanton *et al.*, 2003; Steen and Porter, 2003) and in milk from cows grazing at higher altitude, an effect that has been attributed to higher concentrations of PUFAs in alpine plants (Collomb *et al.*, 2002a,b); this response is lost as grass matures over a grazing season (Auldist *et al.*, 2002); feeding concentrates generally reduce milk CLA content in grazing cows (Stockdale *et al.*, 2003); levels of CLA can be enhanced by including

vegetable oils in the diet (Chouinard *et al.*, 2001; Dhiman *et al.*, 2000; Kelly *et al.*, 1998a; Lock and Garnsworthy, 2002), but greater responses occur when supplements of fish or marine oils are fed (Chilliard *et al.*, 2001; Chouinard *et al.*, 2001; Offer *et al.*, 1999, 2001; Whitlock *et al.*, 2002).

The effect of diet on the levels of CLA in milk and tissues has been reviewed extensively (Bauman *et al.*, 2001; Chilliard *et al.*, 2001; Griinari and Bauman, 1999; Lawson *et al.*, 2001; Stanton *et al.*, 2003). Nutritional strategies for enrichment of RA concentrations of ruminant foods can be grouped into four broad categories relative to potential modes of action: (1) provision of lipid substrates for ruminal VA and RA synthesis, (2) dietary factors that induce changes in microbial populations involved in BH either directly or via changes in rumen environment, (3) diets that provide both lipid substrates and induce changes in ruminal BH, and (4) feeding supplements of rumen-protected CLA and VA. A clear distinction among these approaches often is not possible because responses are usually related to more than a single factor. This section considers the impact of dietary changes on lipid metabolism in the rumen, because the effects of nutrition on CLA content of milk and meat can be related to the effects on ruminal BH and more specifically on the amounts of individual BH intermediates available for absorption. Griinari and Shingfield (2002) also argue that a simple description of dietary factors influencing CLA content in meat and milk does not provide sufficient insight for the development of nutritional strategies that optimize ruminal VA formation that is the major source of RA in ruminant foods, and therefore, *in vivo* assessments of ruminal BH are required.

Measurements of BH *in vivo* are relatively limited and can be summarized as studies examining the effect of forage type (Lee *et al.*, 2003), level of concentrate in the diet (Kalscheur *et al.*, 1997a; Kucuk *et al.*, 2001; Piperova *et al.*, 2002), lipid supplements (Duckett *et al.*, 2002; Kalscheur *et al.*, 1997b; Lock and Garnsworthy, 2002; Shingfield *et al.*, 2003), or the interaction between concentrate level and amount of oil in the diet (Sackmann *et al.*, 2003).

Because the metabolism of *trans*-18:1 to stearic acid is thought to be the rate-limiting step in complete BH (Keeney, 1970), the effects of diet on lipid metabolism in the rumen tend to have a more pronounced effect on the amount and relative proportions of *trans*-18:1 leaving the rumen than on other BH intermediates including isomers of CLA. With respect to enhancing RA content of ruminant foods, it could be argued that the most important aspects of dietary effects on ruminal BH relate to those on VA and the factors that regulate its synthesis.

In a comparison, Lee *et al.* (2003) reported that ruminal synthesis of RA and VA was higher in steers fed red or white clover silage than grass silage. However, dry matter intakes were markedly different among experimental

silage diets, so it is difficult to establish whether the positive effects on ruminal CLA and VA synthesis reflect the higher PUFA content, greater intake potential (and associated changes in digestion kinetics), or both attributes of legume silages. Relatively few studies have examined the effects of different forage sources on ruminal BH. Indirect comparisons of milk fatty acid composition have shown that VA is the predominant *trans*-18:1 from grass silage–based diets (Offer *et al.*, 1999), but *trans*-10 18:1 is the main isomer when corn silage is fed (Offer *et al.*, 2001). These findings suggest that the higher levels of starch, lower amounts of fiber, or both attributes of corn compared with grass silage promote shifts in BH pathways towards *trans*-10 18:1 formation at the expense of VA. Forage conservation method also appears to be important, following the observation that the rate and extent of BH of linoleic and α-linolenic acid *in vitro* is higher for fresh or ensiled grass than dried hay (Boufaïed *et al.*, 2003). However, whether this is a direct effect on BH per se or related to changes in the rate of lipolysis of forage lipids is unclear.

Increases in the amount of concentrate in the diet from 40 to 75% of DM decrease the reduction of *trans* monoenoic acids to stearic acid in the rumen, a change that also is associated with a lower ruminal pH level (Kalscheur *et al.*, 1997b). Adding a buffer to the diet normalized ruminal pH level (6.15 for high-forage diets; 6.02 vs. 5.83 for buffer vs. no buffer in high-concentrate diets) and decreased duodenal *trans*-18:1 flow (61 vs. 57 and 120 vs. 66 g/day for low- and high-concentrate diets, respectively). Further analysis of duodenal digesta from this experiment (Piperova *et al.*, 2002) indicates that high levels of concentrate increased ($P < .05$) all of the *trans* monoenes except *trans*-11 and *trans*-16. The most marked change for high-concentrate diets without buffer was seen in the flow of *trans*-10 18:1, which was fourfold higher compared with the high-forage diets. When buffer was added to the high-concentrate diet, flows of *trans*-9, *trans*-12, *trans*-13/14, and *trans*-15 monoenoic acids were comparable to the high-forage diet. Interactions of forage level and buffer effects were observed for *trans*-12, *trans*-13 + 14 monoenes, and total *trans* fatty acids. Amounts of CLA isomers were much lower compared with the *trans* monoenes, and the effects were highest for the high-concentrate diet without buffer; duodenal flows of *trans*-10, *cis*-12 18:2 increased fourfold, and those of RA, as well as *trans, trans* isomers were doubled ($P < .01$). Changes in the synthesis of individual BH intermediates associated with feeding high levels of concentrates were proposed to be mediated by the effects on ruminal pH. Presumably, changes in ruminal pH were associated with alterations in the balance of the growth and proliferation of specific bacteria capable of BH.

Measurements of duodenal fatty acid flows in the experiment of Kalscheur *et al.* (1997a) are consistent with concentrations of *trans*-10 18:1

in milk being increased fivefold in cows offered a high-concentrate diet supplemented with unsaturated fatty acids (Griinari *et al.*, 1998). Levels of *trans*-10 18:1 were comparable between high-concentrate diets supplemented with saturated fatty acids and high-forage diets containing either saturated or unsaturated fatty acids, indicating that shifts toward the formation of *trans*-10 18:1 were related to two factors: an altered rumen environment and a source of PUFA in the diet.

The effects of concentrate in the diet have also been examined in sheep. Flows of VA decreased linearly from 8.3 to 5.5 g/day as the proportion of forage in the diet increased from 18 to 60% of diet dry matter but increased inexplicably when diets contained proportionately 0.73 forage dry matter (Kucuk *et al.*, 2001). However, the variable addition of soybean oil to ensure diets contained 6% fat resulted in differences in dietary forage:concentrate ratio being confounded with oil supplementation so higher forage diets contained greater amounts of added oil. In spite of these concerns, high levels of concentrate in the diet also were shown to inhibit the final reduction of *trans*-18:1 to stearic acid; unfortunately, the occurrence of *trans*-18:1 other than VA was not reported. High levels of concentrate in the diet were also reported to reduce the formation of RA and increase the synthesis of *trans*-10, *cis*-12 CLA. Daniel *et al.* (2004) reported a near disappearance of VA, associated with large increases in *trans*-10 18:1, in abomasal digesta of lambs in response to a change in diet from dehydrated grass pellets to high-concentrate diets.

Increases in *trans*-18:1 formation also have been shown when diets are supplemented with vegetable oils. Kalscheur *et al.* (1997b) reported that duodenal flow of *trans*-18:1 was increased from 64 g/day for a control diet to 287 and 295 g/day when 500 ml of high oleic or high linoleic acid sunflower oils, respectively, were included in the diets. Unfortunately, flows of CLA or individual *trans*-18:1 isomers were not determined. In another study, duodenal flows of monoenoic and CLA isomers in steers fed corn silage, high oil corn silage, or corn silage supplemented with corn oil were reported (Duckett *et al.*, 2002). Under these feeding conditions, VA was the major BH intermediate for corn silage and high oil corn silage, but supplementing conventional corn silage with an equivalent amount of oil provided by the high oil corn silage enhanced *trans*-10 and *trans*-12 18:1 formation but had no effect on VA production. Thus, free oil was a more potent modifier of rumen BH in this experiment than the same oil contained in the matrix of the cereal grain.

More dramatic inhibition of the reduction of *trans*-18:1 in the rumen was observed when fish oil was included in the diet (Wonsil *et al.*, 1994). Studies have shown that feeding 250 g of fish oil/day to cows fed grass silage–based diets decreased ruminal outflow of stearic acid by 83% (Shingfield *et al.*,

2003) and enhanced the flow of most *trans* monoenes by twofold to fourfold, whereas *trans*-10 and *trans*-11 were increased by sevenfold and sixfold, respectively. Interestingly, whereas increased flows were observed also for nonconjugated dienes (*trans*-11, *cis*-15 increased nearly sixfold), both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated isomers were decreased by feeding fish oil because of associated reductions in dry matter intake. This suggests that fish oil is a more potent inhibitor of bacteria involved in the hydrogenation of *trans*-18:1 and 18:2 than those responsible for the formation of conjugated dienes. Furthermore, the inhibition of complete BH of dietary PUFAs and accumulation of VA in the rumen could account for virtually all of the increase in milk RA content when fish oil was included in the diet.

Not only are alterations in BH dependent on the amount or type of lipid supplement or through changes in the basal diet, but a combination of factors are involved. For example, the production of BH intermediates in response to sunflower oil supplements was shown to depend on the proportion of concentrate in diet (Sackmann *et al.*, 2003). Duodenal flows of RA were higher in steers when supplements of sunflower were included in diets containing Bermuda hay forage at 12 or 24% of diet dry matter than 36% forage (0.16 and 0.21 vs. 0.07 g/day, respectively). Conversely, increases in the proportion of forage in the diet reduced *trans*-10, *cis*-12 CLA production (0.40, 0.26, and 0.22 g/day) and enhanced *cis*-11, *trans*-13 CLA formation (0.02, 0.04, and 0.06 g/day). Even though the composition of the basal diet altered the relative proportions of CLA isomers in response to sunflower oil, much greater changes were observed for duodenal flows of *trans*-18:1 BH intermediates. Increases in the proportion of concentrate in the diet reduced ruminal synthesis of VA (11.8, 7.0, and 3.5 g/day for diets containing 64, 76, and 88% concentrate dry matter, respectively), *trans*-9 18:1 (2.1, 2.3, and 0.7), and *trans*-12 18:1 (2.5, 2.5, and 1.3) but caused a marked increase in *trans*-10 18:1 (15.5, 29.8, and 41.4).

Griinari and Shingfield (2002) suggest that ruminal VA formation is dependent on three interdependent processes: (1) substrate supply, (2) inhibition of *trans*-18:1 reductase, and (3) prevention of a shift in ruminal BH toward *trans*-10 18:1 at the expense of *trans*-11 18:1 (Figure 6). This hypothesis, termed the "biohydrogenation balance model," attempted to characterize the impact of diet on the CLA content of ruminant foods by taking into account the effects of all three processes on ruminal BH simultaneously. The authors suggested that substrate supply has a typically permissive role in determining the extent of *trans*-18:1 fatty acid accumulation in the rumen in response to the other two processes, so the balance between *trans*-18:1 reductase inhibition and induction of the shift toward *trans*-10 18:1 at the expense of VA regulates the magnitude of the overall response.

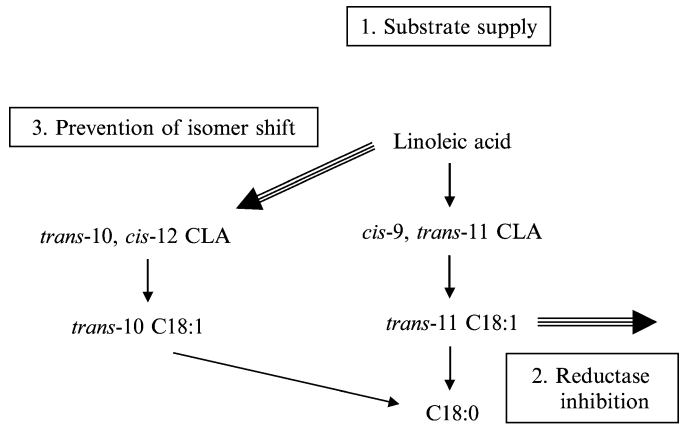


FIG. 6 Schematic of the "biohydrogenation balance model." (Used, with permission, from Griinari and Shingfield, 2002.)

To summarize the impact of diet, complete BH to stearic acid is most extensive when animals are fed diets containing high amounts of ensiled forages. Incomplete reduction to stearic acid, associated with the accumulation of BH intermediates, most notably *trans* 18:1, arises when diets contain high amounts of readily available unsaturated fatty acids, low amounts of fiber, or high levels of starch or cause low ruminal pH. Fish and marine oils are far more potent inhibitors of *trans*-18:1 reduction in the rumen than other sources of unsaturated fatty acids (Chilliard *et al.*, 2001; Offer *et al.*, 2001; Whitlock *et al.*, 2002). Changes from basal diets by a single dietary parameter in isolation have little effect on BH; simultaneous changes in interdependent processes are required to have an impact. It is this that explains the difficulties in predicting the effects of changes in the diet on the amount of ruminal BH intermediates available for absorption.

## III. CLA SYNTHESIS BY NON-RUMINAL ORGANISMS

Alternatives to using ruminant foods to provide CLA in the diet are of great interest to the food-processing industry, perhaps most so in dairy processing. Sieber *et al.* (2004) reviewed the impact of microbial cultures on CLA in dairy products. Several strains of *Lactobacillus, Propionibacterium, Bifidobacterium,* and *Enterococcus* are able to form CLA from linoleic acid; lactic acid bacteria and propionibacteria appear to show promise to increase CLA during ripening of cheese. Presently, data are not convincing that this

approach significantly increases CLA content of dairy foods; further, effects are much smaller than can be accomplished by manipulating the animals' diets.

Of interest is a unique alternative biosynthetic pathway for CLA. Ogawa *et al.* (2001) reported that a strain of *Lactobacillus acidophilus,* under microaerobic conditions, produced 10-hydroxy-*cis*-12-octadecenoic acid and 10-hydroxy-*trans*-12-octadecenoic acid as intermediates in the synthesis of *cis*-9, *trans*-11 and *trans-9*, *cis*-11 18:2. The conversion was induced by presence of linoleic acid, and a high yield of CLA was reported. Hudson *et al.* (1998, 2000) showed that lactic acid bacteria, including *Lactobacillus, Pediococcus,* and *Streptococcus* species, are the major unsaturated fatty acid hydrating bacteria in the rumen, converting oleic acid to 10-hydroxy stearic acid and linoleic acid to 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9 octadecenoic acid. Thus, potentially, CLA may be produced also in the rumen from linoleic acid by pathways other than the classic isomerase described by Kepler *et al.* (1966).

Finally, dairy products may be enriched with naturally occurring CLA by fat fractionation procedures. Fractionation of anhydrous milk fat by a supercritical carbon dioxide system (Romero *et al.*, 2000) or by controlled cooling and agitation (O'Shea *et al.*, 2000) resulted in both cases in a more than 60% increase in the CLA content as compared to the parent fat; also concentrations of PUFA and VA were increased.

## IV. ENDOGENOUS SYNTHESIS OF CLA

### A. BACKGROUND

As is outlined in the following section, it is now apparent that endogenous synthesis of RA occurs in most, if not all, animal species. The classic studies of Mahfouz *et al.* (1980) and Pollard *et al.* (1980) showed independently that positional isomers of trans-octadecenoic acids are desaturated by the enzyme Δ-9 desaturase in rat liver microsomal systems. All *trans* monoenes, Δ-4 to Δ-13, except Δ-8, 9, and 10, were substrates, with products being *trans*-Δ-x, *cis*-9 dienes. The rate of Δ-9 desaturation increased as the *trans* bond was removed further from the Δ-9 position, so that *trans*-Δ-4 and Δ-13 monoenes were most rapidly desaturated (Mahfouz *et al.*, 1980). The *trans*-5, *cis*-9 18:2 was isomerized rapidly to the cis, cis diene without changing bond positions; *trans*-4, *cis*-9 18:2 was isomerized similarly, but at a slower rate (Mahfouz *et al.*, 1980). Significant amounts of some of the *cis*/*trans* dienes were desaturated further at Δ-6, to yield *cis, trans, cis* trienes (Pollard *et al.*, 1980).

The end-products of rumen microbial metabolism of greatest interest with regard to CLA metabolism in the body are the various CLA isomers and the *trans*-11 monoenoic acid VA. The latter, being a substrate for Δ-9-desaturase, is converted to RA in animal tissues. Hay and Morrison (1970) reported the distribution of monoenoic isomers of milk fat, and the content of various CLA and other 18:2 isomers in the fat of ruminant products has been summarized by Parodi (2003).

## B. CHARACTERISTICS OF Δ-9-DESATURASE

Although we refer to the enzyme as Δ-9-desaturase because it is active with numerous acyl-coenzyme A (acyl-CoA) substrates, its most common substrate is stearic acid, so it is most often identified as stearoyl-CoA desaturase (EC 1.14.99.5). The significance of endogenous synthesis as a source for RA emphasizes the critical role of Δ-9-desaturase in the production of RA in milk and meat fat in ruminants and in the conversion of dietary VA to RA in other species. Δ-9-Desaturase is a key regulatory enzyme for the biosynthesis of monounsaturated fatty acids that are in turn used for the synthesis of triacylglycerols, phospholipids, and cholesterol esters. Understanding of this enzyme in ruminants is limited, with current knowledge coming predominantly from investigations with rodents and rodent cell lines.

The oxidative reaction catalyzed by Δ-9-desaturase involves cytochrome $b_5$, NAD(P)-cytochrome $b_5$ reductase and molecular oxygen, and the CoA ester of fatty acids is required as the substrate (Ntambi, 1995). Thus, the CoA ester of VA is the substrate for formation of RA. Preferred substrates for Δ-9-desaturase are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively (Ntambi, 1999). Δ-9-Desaturase has no known allosteric or feedback inhibition involving its substrates or products. The regulation of this enzyme has been investigated extensively in rodent liver, and results indicate that gene expression and amount of enzyme is regulated by dietary factors such as glucose and PUFA and by hormones such as insulin, glucagon, and thyroid hormone (Ntambi and Miyazaki, 2004). Δ-9-Desaturase gene expression is down-regulated by both PUFA and *trans*-10, *cis*-12 CLA, but RA has no effect (Choi *et al.*, 2000; Lee *et al.*, 1998; Ntambi and Miyazaki, 2004). The point of regulation for the enzyme is gene transcription, which is effective because of the short half-life of the Δ-9-desaturase protein (~4 hours; Ozols, 1997). In mice, a significant proportion of the effects of leptin on metabolism may be mediated by inhibition of Δ-9-desaturase expression (Ntambi and Miyazaki, 2004). Interestingly, sterculic oil does not affect Δ-9-desaturase gene or protein expression, but it directly inhibits the activity of Δ-9-desaturase, possibly by a turnover-dependent

reaction (Gomez *et al.*, 2003). Both RA and *trans*-10, *cis*-12 CLA were reported to influence stearoyl-CoA desaturase activity in human breast cancer cell lines; however, mechanisms apparently differed between cell lines (Choi *et al.*, 2002). Both CLA isomers reduced stearoyl-CoA desaturase protein in MDA-MB-231 cells, whereas in MCF-7 cells, both isomers directly inhibited the enzyme activity. These results differ from other reports on effects of RA on desaturase activity; they have not been repeated and perhaps were unique to these cell types.

Three isoforms of the Δ-9-desaturase gene have been characterized in mice, whereas two have been characterized in rats and only one isoform has been identified in humans and ruminants (Ntambi and Miyazaki, 2004). Relatively few studies have involved ruminants or examined the regulation of Δ-9-desaturase in mammary tissue of any lactating mammal. Beswick and Kennelly (2000) reported that recombinant bovine growth hormone and bovine growth hormone–releasing factor decreased Δ-9-desaturase mRNA in adipose tissue, but not mammary tissue, of lactating cows. Δ-9-Desaturase activity has been reported in bovine milk microsomes (McCarthy *et al.*, 1965) and in adipose tissue, muscle, intestine, liver, and mammary gland of ruminants (Bickerstaffe and Annison, 1969, 1970; Chang *et al.*, 1992; St. John *et al.*, 1991). Abundance of mammary tissue Δ-9-desaturase decreases when milk fat depression is induced by diet (Peterson *et al.*, 2003a) or by supplements of *trans*-10, *cis*-12 CLA (Baumgard *et al.*, 2002). Regulation of this enzyme in mammary tissue probably involves sterol response element binding protein-1 (SREBP-1). 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 (Clarke, 2001; Horton *et al.*, 2002; Shimano, 2001). The addition of *trans*-10, *cis*-12 CLA to bovine mammary epithelial cells was shown to reduce the proteolytic activation of SREBP-1 (Peterson *et al.*, 2003b).

Ward *et al.* (1998) reported highest expression of Δ-9-desaturase mRNA in adipose tissue, liver, and mammary gland of lactating sheep, and expression was decreased by 80% in adipose tissue of late pregnant and lactating animals, a time when lipogenic activity is increased in mammary gland and decreased in adipose tissue (Bauman and Currie, 1980). Whereas Ward *et al.* (1998) reported significant Δ-9-desaturase mRNA expression in liver, St. John *et al.* (1991) found no enzymatic activity in liver of steers fed a diet high in corn. The same research group (Chang *et al.*, 1992) reported inconsistent desaturase activity (one of four animals) in liver of cattle fed a high-corn diet, whereas activity in liver was detected when a diet containing 8% oil from high oleate sunflower seeds was fed. The authors indicated that the unsaturated sunflower oil was extensively BH in the rumen, thus increasing the amount of stearic acid absorbed. Desaturase activity was also increased

in adipose tissue and intestinal mucosa and was induced sixfold ( $P < .05$) in muscle. Although low desaturase activity in ruminant liver is consistent with low liver uptake of stearic acid (Bell, 1981), further studies are warranted to document induction of Δ-9-desaturase in ruminant tissues under conditions of high amounts of stearic acid absorption. Desaturase index has been shown to be different among adipose depots of sheep (Palmquist *et al.*, 2004) and growing Jersey cattle (Leat, 1975) but was not different in subcutaneous adipose tissues of two beef breeds, Angus and American Wagyu (Cameron *et al.*, 1994). A number of studies have reported breed differences in the CLA content of milk fat (Dhiman *et al.*, 2002; Lawless *et al.*, 1999; White *et al.*, 2001; Whitlock *et al.*, 2002), which may reflect differences in desaturase index among breeds. However, these studies have 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 desaturase index in milk fat of dairy cows, consistent with the suggestion that the activity of Δ-9-desaturase is higher in Holstein than Jersey mammary tissue (Beaulieu and Palmquist, 1995). However, if breed differences exist, they are minor compared with the effect of diet and individual animal variation on the CLA content of milk fat and desaturase index (Bauman *et al.*, 2003).

### C. ENDOGENOUS SYNTHESIS OF RA IN RUMINANTS

Griinari and Bauman (1999) proposed that endogenous synthesis could be an important source of the RA found in milk fat of ruminants, based on its transient occurrence as an intermediate in ruminal BH, as well as the observation that increases in the RA content of milk fat occurred when the dietary supply of linolenic acid was increased. As previously discussed, VA is an intermediate in the BH of both linoleic and linolenic acids and is the major unsaturated BH product passing from the rumen. Strong support for the hypothesis of endogenous synthesis was gained by abomasal infusion of VA (12.5 g/day to lactating dairy cows) that resulted in a 31% increase in milk fat content of RA (Griinari *et al.*, 2000). Although this study suggested that endogenous synthesis was a source of milk fat CLA, its contribution to total RA in milk fat was unknown.

Researchers have used one of two approaches to quantify endogenous synthesis. The first involves inhibiting the Δ-9-desaturase, thereby inhibiting conversion of VA to RA. Using abomasal infusion of sterculic oil to inhibit Δ-9-desaturase activity, combined with monoene/saturate pair ratios to correct for the extent of inhibition, Griinari *et al.* (2000) estimated that a minimum of 64% of RA in their milk fat samples was derived from desaturation of VA. Further experiments using sterculic oil also indicated that the

majority of the RA in milk fat was derived from endogenous synthesis via Δ-9-desaturation of VA. Corl *et al.* (2001) estimated that endogenous synthesis contributed more than 78% of milk fat RA when cows were fed a total mixed ration of hay and concentrate. Endogenous synthesis contributed an even greater extent when cows grazed fresh pasture, with more than 91% of RA in milk fat synthesized endogenously (Kay *et al.*, 2004).

A second approach to quantify endogenous synthesis of RA in milk fat uses indigestible markers and estimates ruminal outflow of RA. By comparing ruminal outflow of RA with milk output of RA, the maximum proportion of RA derived from ruminal production can be estimated, with endogenous synthesis representing the remainder. Assumptions and limitations to this approach and to inhibiting Δ-9-desaturase directly have been discussed (Bauman *et al.*, 2003). Piperova *et al.* (2002) fed high- and low-fiber diets with or without buffer and in all cases found that duodenal supply of RA was a very small percentage of the amount secreted in milk, whereas duodenal flow of VA provided more than adequate amounts of precursor. Calculations from their data show that endogenous synthesis accounted for more than 93–97% of milk RA, and this represented 20–33% of the duodenal supply of VA. Similar results have been reported by others. By extrapolating results from non-lactating dairy cows to lactating cattle on the basis of feed intake, Lock and Garnsworthy (2002) estimated that endogenous synthesis accounted for over 80% of the RA in milk fat when cows were fed a grass silage diet with concentrates differing in their content of linoleic and linolenic acids. Shingfield *et al.* (2003) used ruminal outflow measurements to calculate that endogenous synthesis accounted for more than 74% of the RA in milk fat when a grass silage plus concentrate diet was fed with or without fish oil.

Estimating the extent of endogenous RA synthesis in growing ruminants is inherently more difficult because RA in body fat accumulates throughout the lifespan of the animal. Nevertheless, ruminal output of VA ranged from 27- to 69-fold greater than output of RA for sheep fed diets that varied in forage content and supplementation with soybean oil (Kucuk *et al.*, 2001). Similar results were shown in cattle fed corn-based finishing rations with or without a corn oil supplement, in which ruminal outflow of VA ranged from 39- to 61-fold greater than rumen outflow of RA (Duckett *et al.*, 2002). When beef heifers were fed various diets (hay–concentrate mixtures, grazing pasture, and plant oil supplements), in all cases ruminal outflow of VA was substantially greater than outflow of RA (Carter *et al.*, 2002; Lake *et al.*, 2002; Scholljegerdes *et al.*, 2002). The only study to estimate endogenous synthesis of RA in growing ruminants used a mathematical modeling approach and estimated that 45–95% of RA in muscle and adipose tissues of lambs was synthesized endogenously (Palmquist *et al.*, 2004). They also

estimated that desaturation of VA ranged from 11 to 22% and concluded that the proportion of RA synthesized endogenously was inversely related to the amount of RA absorbed from the intestine and found in the tissues.

The relationship between substrate and product for Δ-9-desaturase is reflected by the desaturase index, defined as [RA ÷ (RA + VA)]. Various approaches to calculating desaturase index in milk fat are discussed by Kelsey *et al.* (2003). In the study by Corl *et al.* (2001), the desaturase index was 0.23 for the hay and concentrate diet and 0.20 when the diet was supplemented with PHVO. Kay *et al.* (2004) reported a desaturase index of 0.25 for the pasture diet and 0.22 when the diet was supplemented with sunflower oil. Piperova *et al.* (2002) observed desaturase indices for high- and low-fiber diets of 0.40 and 0.35, respectively. Shingfield *et al.* (2003) reported desaturase indices of 0.18 and 0.15 with a grass silage diet without or supplemented with fish oil; these values are probably lower than others because the analytical methods accounted for minor CLA isomers that typically co-elute with RA or because of inhibition of Δ-9-desaturase by the long-chain PUFA from the fish oil supplement. The desaturase index, as defined earlier, should approximate the proportion of VA desaturated in the tissues. A summary of endogenous RA synthesis estimates and the proportion of VA desaturated in the tissues is in Table III.

Although diet is the major determinant of milk fat RA content, there is also a twofold to threefold range in milk fat content of RA among individual cows within a herd consuming the same diet (Bauman *et al.*, 2003). A similar level of variation also has been shown in the desaturase index, with a several-fold range among cows (Kelsey *et al.*, 2003; Lock and Garnsworthy, 2002, 2003; Peterson *et al.*, 2002a). Peterson *et al.* (2002a) demonstrated a consistency in the hierarchy among cows in desaturase index over time when cows were fed the same diet and when cows were switched between diets. In the largest study of this type, Kelsey *et al.* (2003) demonstrated that the variation in milk fat content of RA, and the desaturase index, was about threefold among individuals consuming the same diet. The effect of breed (Holstein vs. Brown Swiss), parity, and days in milk had no relationship to the individual variation in desaturase index, and neither did milk yield, milk fat percentage, or milk fat yield (Kelsey *et al.*, 2003; Lock *et al.*, 2003).

Overall, investigations in both lactating and non-lactating ruminants have shown that the major source of RA in milk fat and adipose tissue is endogenous synthesis, with the precursor being VA derived from rumen production. The relatively constant ratio observed between VA and RA in milk fat reflects the substrate–product relationship for Δ-9-desaturase; therefore, successful approaches to increase the milk fat content of RA will involve enhancing rumen output of VA and increasing tissue activity of Δ-9-desaturase, as discussed in a following section.

TABLE III
QUANTITATIVE STUDIES OF ENDOGENOUS CLA SYNTHESIS IN LACTATING COWS

| Source | Diet | Duodenal flow (g/day) | | Content in milk fat (%) | | Endogenous (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | VA | RA | VA | RA | Desaturase inhibition[a] | Rumen outflow[b] | VA to RA (%)[c] |
| | | | | (%) | | | | |
| Griinari *et al.*, 2000 | Hay/concentrate TMR | — | — | 1.4 | 0.43 | 64 | — | <20 |
| Corl *et al.*, 2001 | Hay/concentrate TMR | — | — | 2.18 | 0.65 | 78 | — | 23.4 |
| | + partially hyd. veg. oil | — | — | 3.03 | 0.76 | 78 | — | 16.4 |
| Kay *et al.*, 2004 | Pasture | — | — | 3.56 | 1.21 | 91 | — | 24 |
| | + sunflower oil | — | — | 4.20 | 1.16 | 91 | — | 20 |
| | | | | g/day | | | | |
| Piperova *et al.*, 2002 | High forage | 21.4 | 0.35 | 10.9 | 7.3 | — | 97 | 39 |
| | High forage + buffer | 20.7 | 0.3 | 11.0 | 6.6 | — | 97 | 37 |
| | Low forage | 34.6 | 0.5 | 14.4 | 7.6 | — | 96 | 34 |
| | Low forage + buffer | 21.5 | 0.2 | 9.7 | 6.0 | — | 98 | 38 |
| Shingfield *et al.*, 2003 | Grass silage/concentrate | 17 | 2.9 | 12.8 | 2.8 | — | 39 | 8 |
| | + fish oil | 121 | 2.1 | 50.9 | 9.0 | — | 86 | 13 |

*Note:* VA, vaccenic acid; RA, rumenic acid.
[a]Endogenous synthesis estimated by sterculic acid inhibition of Δ-9-desaturase combined with saturate/monoene pair ratios to correct for extent of inhibition.
[b]Calculated from respective authors' data; endogenous synthesis estimated using markers to estimate rumen outflow and assuming that 60% of duodenal fatty acid flow is incorporated into milk fat (80% absorption [Palmquist, 1991]; 75% of absorbed fatty acids incorporated into milk fat [Palmquist and Mattos, 1978]).
[c]Percentage of VA desaturated to RA. See text for calculation.

The second most prevalent CLA isomer in ruminant fat is *trans*-7, *cis*-9 CLA, representing 3–16% of total CLA in ruminant fat (Corl *et al.*, 2002; Parodi, 2003; Piperova *et al.*, 2000, 2002; Shingfield *et al.*, 2003; Yurawecz *et al.*, 1998). A number of the studies previously described have determined the source of *trans*-7, *cis*-9 CLA in ruminant fat. Corl *et al.* (2002) showed that the *trans*-7, *cis*-9 CLA in milk fat was derived almost exclusively from endogenous synthesis by using both sterculic acid and *trans*-10, *cis*-12 CLA to inhibit Δ-9-desaturase; they also found that *trans*-7, *cis*-9 CLA concentration in rumen fluid was very low and at the limit of detection. Similarly, Piperova *et al.* (2002) found that virtually all of the *trans*-7, *cis*-9 CLA in milk fat was produced post-ruminally. As mentioned previously, *trans*-7 18:1, a minor BH intermediate in ruminal contents, also is a substrate for Δ-9-desaturase (Mahfouz *et al.*, 1980; Pollard *et al.*, 1980).

## D. ENDOGENOUS SYNTHESIS OF RA IN HUMANS AND OTHER SPECIES

In addition to ruminants, the conversion of dietary VA to RA occurs in humans. Fogerty *et al.* (1988) recognized that dietary VA might be converted to RA in human tissues, quoting earlier work of Pollard (1980) with Δ-9-desaturase in rat liver microsomes. However, based on results from their study, they concluded that nondietary RA came from "free radical isomerization of linoleic acid *in vivo*." Parodi (1994) also recognized the possibility of endogenous synthesis and suggested further that another non-dietary source of CLA could be production by bacteria in the digestive tract. Chin *et al.* (1994) examined this and concluded that intestinal bacteria were capable of converting linoleic acid to CLA based on comparisons between conventional and germ-free rats.

Emken *et al.* (1986) fed deuterium-labeled VA as triacylglycerol to two young adult male subjects and found no evidence in plasma lipids of VA desaturation. Subsequently, they reanalyzed the samples from one subject and reported a CLA enrichment of about 30% (Adlof *et al.*, 2000). Salminen *et al.* (1998) showed that humans consuming a diet high in trans fatty acids (25% of dietary fatty acids) had higher serum concentrations of CLA than those consuming diets low in trans fatty acids. The addition of VA to human mammary and colon cancer cell lines resulted in an increased cell content of RA (Miller *et al.*, 2003). Turpeinen *et al.* (2002) used slope response to increasing dietary VA to estimate the extent of VA desaturation to RA in humans. Thirty healthy subjects consumed a baseline diet rich in oleic acid for 2 weeks, followed by diets containing 1.5, 3.0, or 4.5 g of VA/day for 9 days. Test diets contained no CLA. The change in RA in the serum

very-low-density lipoprotein (VLDL) triacylglycerol was plotted versus the change in VA plus the change in RA in the serum VLDL triacylglycerol. The slope of the regression indicated that 19% of the supplementary VA was converted to RA as a mean response. Based on this work, Parodi (2003) has suggested that CLA intake multiplied by 1.4 would provide an estimate of the effective physiological dose of CLA derived from ruminant products.

Endogenous synthesis of RA from VA also has been shown in other species including rats, mice, and pigs. In a study that compared CLA-enriched butter to commercial CLA sources as anticancer agents in rats, Ip *et al.* (1999) found that when provided at equal dietary CLA levels, butter was a more effective agent to suppress proliferation of mammary terminal end bud cells and mammary tumor yield. Rats consuming butter accumulated twice as much RA in the mammary fat pad and other tissues as those fed free CLA, and authors suggested that this was due to the VA in butter being converted to RA. This was confirmed when feeding rats increasing amounts of pure VA resulted in a progressive increase in the tissue concentration of RA (Banni *et al.*, 2001). Further, increasing dietary supply of a VA-enriched butter resulted in a dose-dependent decrease in mammary tumors and an increase in the accumulation of RA in liver, plasma, and mammary fat pad, with the ratio of VA to RA in the mammary fat pad approaching 1:1 (Corl *et al.*, 2003). Inhibiting Δ-9-desaturase in the same rodent mammary cancer model by feeding sterculic oil reduced the accumulation of RA in tissues with a corresponding reduction in the suppression of mammary premalignant lesions; there was a 39% reduction in the RA content of the mammary fat pad with the addition of sterculic oil to diets containing 1.6% VA (Lock *et al.*, 2004). Considering that RA has been shown to be effective in reducing plasma cholesterol and cholesterol-induced atherogenesis, it seems logical that dietary VA supplied by dairy products also may have beneficial effects on variables associated with increased risk for atherosclerosis, and that this will relate to its use for endogenous synthesis of RA in a manner similar to the ability of dietary VA to reduce mammary cancer risk.

Santora *et al.* (2000) showed that 50% of VA in tissues of mice was desaturated to RA; increasing intake of unsaturated corn oil inhibited the desaturase and decreased desaturation by 30%. VA fed to lactating mice increased VA and RA in the plasma, tissue lipids, and milk lipids of the dams, and in liver of the suckling pups (Loor *et al.*, 2002). However, RA was not found in the tissue lipids of the nursing pups. Finally, Gläser *et al.* (2000) reported that feeding partially hydrogenated vegetable fat as a source of VA to fattening pigs increased CLA content (0.44% of total fatty acids) of the back fat.

## V. CONCLUDING SUMMARY

Because of its potential to improve human health, there is great interest to increase the amount of CLA in the human food supply. This has caused a great deal of effort to be expended toward increasing the concentration of CLA, and more specifically RA, in the milk and tissues of ruminant foods because these are the predominant source of CLA in human diets. RA is the predominant CLA isomer present in ruminant products, and the major source of its occurrence is endogenous synthesis via desaturation of VA by Δ-9-desaturase. The central effort of this research has been directed toward improving the understanding of biohydrogenation in the rumen and examining milk and tissue CLA responses to a range of diets. Even though the major metabolic pathways are well documented, the diversity of various BH intermediates in digesta, milk, and tissues indicates the complexity of the BH processes as a whole and the population dynamics of the ruminal bacteria involved. Predicting the outcome of changes in the diet is complicated by the interactions of the ruminal environment, substrate supply, and forms of dietary lipids, all of which influence the BH process simultaneously. Development of nutritional strategies for enhancing the RA content of ruminant foods has been focused on increasing ruminal supply of VA and preventing shifts in BH toward other *trans*-18:1 isomers. The predominant source of *trans*-7, *cis*-9 CLA and RA in ruminant tissues is clearly via endogenous synthesis from *trans*-7 and *trans*-11 C18:1 as precursors, by the actions of Δ-9-desaturase. Other isomers of CLA found in ruminant foods are related directly to their synthesis in the rumen. Given the importance attached to RA, understanding the variation in activity of Δ-9-desaturase in tissues also has become a major focus of research. Research indicates that it is possible through dietary means to increase the concentration of RA in milk by 5- to 10-fold, but the increases reported for tissues appear to be lower. In all cases, use of nutrition to enrich ruminant foods with RA is associated with an unavoidable increase in *trans*-18:1 content. Levels of *trans*-10, *cis*-12 CLA in ruminant foods are extremely low and, therefore, are not significant sources of this isomer in the human diet. Finally, humans and other species also are able to convert VA to RA via Δ-9-desaturase, thereby further increasing availability of RA in the human diet from ruminant products.

This review cites research published to March 2004.

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