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BOARD-INVITED REVIEW: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem[1]

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ABSTRACT: Recent advances in chromatographic identification of CLA isomers, combined with interest in their possible properties in promoting human health (e.g., cancer prevention, decreased atherosclerosis, improved immune response) and animal performance (e.g., body composition, regulation of milk fat synthesis, milk production), has renewed interest in biohydrogenation and its regulation in the rumen. Conventional pathways of biohydrogenation traditionally ignored minor fatty acid intermediates, which led to the persistence of oversimplified pathways over the decades. Recent work is now being directed toward accounting for all possible *trans*-18:1 and CLA products formed, including the discovery of novel bioactive intermediates. Modern microbial genetics and molecular phylogenetic techniques for identifying and classifying microorganisms by their small-subunit rRNA gene sequences have advanced knowledge of the role and contribution of specific microbial species in the process of biohydrogenation. With new insights into the pathways of biohydrogenation now available, several attempts have been made at modeling the pathway to predict ruminal flows of unsaturated fatty acids and biohydrogenation intermediates across a range of ruminal conditions. After a brief historical account of major past accomplishments documenting biohydrogenation, this review summarizes recent advances in 4 major areas of biohydrogenation: the microorganisms involved, identification of intermediates, the biochemistry of key enzymes, and the development and testing of mathematical models to predict biohydrogenation outcomes.

Key words: biohydrogenation, rumen, microorganism, conjugated linoleic acid, modeling

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INTRODUCTION

Fatty acid metabolism in the rumen has a major influence on the fatty acid composition of ruminant meats and milk. Unsaturated fatty acids, including α-linolenic acid (*cis*-9, *cis*-12, *cis*-15-18:3) and linoleic acid (*cis*-9, *cis*-12-18:2), are abundant in grass and certain other ruminant feedstuffs, yet are present at low concentrations in meat and milk. Tissue lipids of ruminants have been known for a long time to be more saturated than those of nonruminants (Banks and Hilditch, 1931). The consumption of dairy products and ruminant meats is often associated with an increased incidence of coronary heart disease in man (Menotti et al., 1999). An important research aim is to optimize the fatty acid content of these products by decreasing their saturated fatty acid content and increasing their n-3 fatty acids

[1]Throughout the manuscript, fatty acids found in plant matter (oleic, linoleic, linolenic, and stearic) are referred to by common name and intermediates of biohydrogenation are referred to by number of carbons:number of double bonds preceded by position and *cis/trans* geometry (if known).

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in particular (Scollan et al., 2001). Furthermore, ruminant products also contain potentially health-promoting CLA, mainly *cis*-9, *trans*-11-CLA. Dietary CLA have been shown in many animal studies to contribute to cancer prevention, decreased atherosclerosis, improved immune response, and altered protein or energy metabolism (Whigham et al., 2000; Belury, 2002; Pariza, 2004; Palmquist et al., 2005). The CLA are present at higher concentrations in ruminant products than in corresponding meats from nonruminants or in vegetable oils (Chin et al., 1992; Lawson et al., 2001), because they are formed in the rumen from dietary linoleic acid. C*is*-9, *trans*-11-CLA is generally considered to be the main health-promoting CLA for human consumption (Pariza, 2004). The *trans*-11-18:1 fatty acid, an intermediate of biohydrogenation (**BH**), is also desirable as a product flowing from the rumen because *trans*-11-18:1 acts as a substrate for the formation of *cis*-9, *trans*-11-CLA in the animal's own tissues (Griinari et al., 2000). The microorganisms present in the rumen are those that effect the transformation from PUFA to SFA (Shorland et al., 1955; Viviani, 1970), a process that has come to be known as BH. The main aim of investigations into ruminal BH is to create healthier ruminant products. This paper reviews recent progress in the field.

HISTORICAL PERSPECTIVE OF BH

When dietary material enters the rumen, it enters a large fermentation vat, where it undergoes a wide range of chemical changes performed by the microbial population (Harfoot, 1978). Lipids are extensively altered in the rumen, resulting in marked differences between the fatty acid profile of lipids in the diet (mostly unsaturated fatty acids) and lipids leaving the rumen (mostly SFA). Ruminal microbes transform lipids entering the rumen via 2 major processes, lipolysis and BH.

Lipids entering the rumen are first transformed by microbial lipases in a process called lipolysis. The microbial lipases hydrolyze the ester linkages in complex lipids, causing the release of fatty acids (Garton et al., 1961; Dawson et al., 1977). After lipolysis, unsaturated fatty acids undergo BH by ruminal microbes. This process (Figure 1) converts the unsaturated fatty acids to SFA via isomerization to *trans* fatty acid intermediates, followed by hydrogenation of the double bonds (Harfoot and Hazlewood, 1988). The rates of lipolysis and BH are dependent on the type and amount of fat delivered to the rumen (Beam et al., 2000) and ruminal pH (Van Nevel and Demeyer, 1996).

The main types of dietary lipids entering the rumen are triglycerides, phospholipids, and galactolipids. Microbial enzymes rapidly hydrolyze triglycerides, as Garton et al. (1958) observed when linseed oil and tung oil were incubated with ruminal contents of sheep (1.0 g/100 mL), with greater than 75% of the total lipid recovered in the form of FFA. Dawson (1959) examined the hydrolysis of phospholipid by incubating lecithin labeled with ^{32}P with sheep ruminal contents or a washed preparation of ruminal microorganisms. There was rapid breakdown of lecithin, and the ^{32}P-label appeared as inorganic phosphorus. Additionally, when ^{14}C-labeled grass was introduced into the sheep rumen, galactolipids from the ^{14}C-labeled grass were rapidly hydrolyzed to FFA and galactosylglycerol (Dawson et al., 1974).

The majority of the hydrolysis of galactolipids and phospholipids is a result of the enzyme activity of ruminal microorganisms (Dawson and Hemington, 1974). However, Faruque et al. (1974) suggested that the hydrolysis of triglycerides and galactolipids from grass was due primarily to plant enzyme activity. Dawson et al. (1977) autoclaved ^{14}C-labeled grass to inactivate plant lipolytic enzymes. The grass was then administered intraruminally to a sheep, and the galactolipids were rapidly hydrolyzed. Additionally, in an in vitro trial, grass was homogenized with rumen fluid taken from sheep that had been given autoclaved grass for 7 d, and the grass galactolipids were rapidly hydrolyzed. The rumen fluid was assumed to be devoid of plant lipases. In the absence of plant lipases, the grass galactolipids were rapidly hydrolyzed. Grass was also homogenized with boiled ruminal fluid to inactivate microbial lipases, and the galactolipids were not hydrolyzed. Therefore, the authors concluded that lipases produced by ruminal microorganisms were mainly responsible for the hydrolysis of ingested plant lipids in the rumen (Dawson et al., 1977). This is not to say that plant lipases are unimportant. Dierick and Decuypere (2002) showed that after storage for 56 d, approximately 25 to 30% of the fat in milled corn, wheat, and barley was lipolyzed, and for milled sorghum and oats the degree of lipolysis was 50 and 65%, respectively. Further research is required in this area to better define the action of plant endogenous lipases external to the rumen.

Anaerovibrio lipolytica is a ruminal bacterium that has been found to produce 2 hydrolytic enzymes. One is a cell-bound esterase and the other is an extracellular lipase (Harfoot, 1978). The lipase shows activity toward triglycerides and esterified fatty acids (Henderson, 1971). Hespell and O'Bryan-Shah (1988) examined the esterase activity of various rumen bacteria, including 30 strains of *Butyrivibrio fibrisolvens*. All were shown to possess some esterase activity, but few species could hydrolyze esters with long-chain fatty acids. Similarly, Fay et al. (1990) identified 74 strains of ruminal bacteria that could hydrolyze ρ-nitrophenylpalmitate with a range of activity.

Early evidence of ruminal BH was observed when linseed oil was incubated with sheep ruminal contents (Reiser, 1951). After incubation, the linolenic acid content of the linseed oil decreased from 30 to 5%, with a concurrent increase in the concentration of 18:2. Shorland et al. (1955) also studied the effect of BH on linolenic acid. They observed a conversion of linolenic acid to 18:2 and 18:1 intermediates, which then terminated with stearic acid. Garton et al. (1958) observed that after the hydrolysis of triglycerides, the fatty acids were

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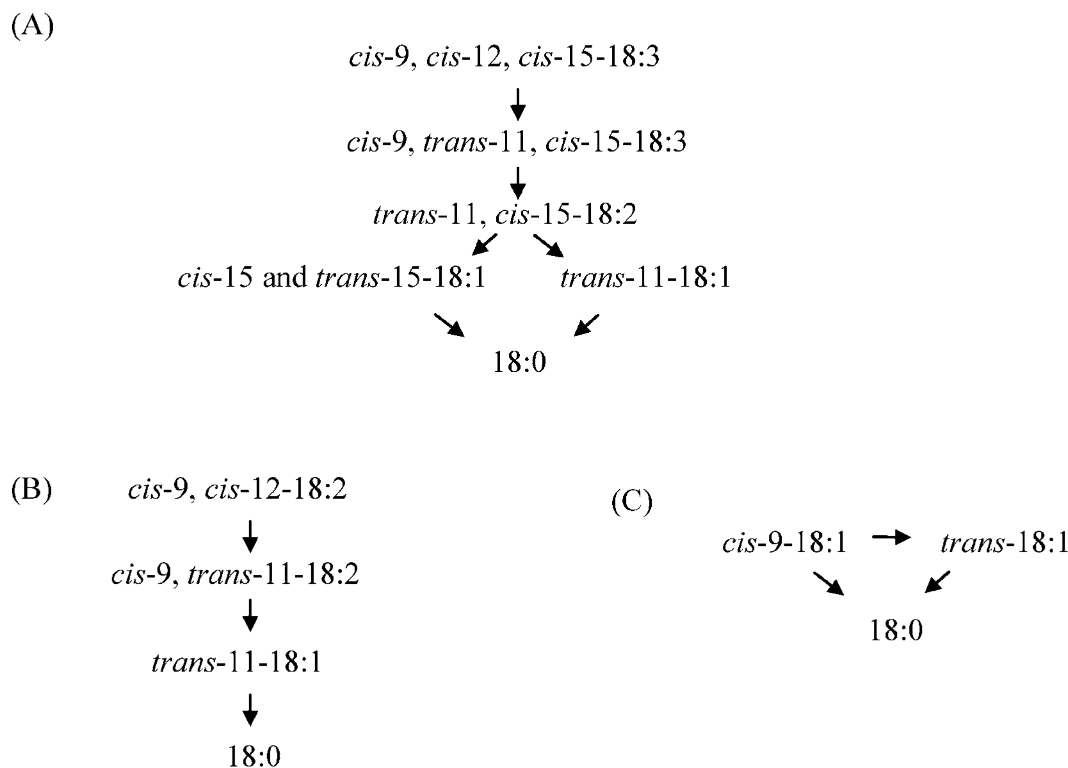

Figure 1. Biohydrogenation pathways of (A) α-linolenic, (B) linoleic, and (C) oleic acids. Adapted from Harfoot and Hazlewood (1988).

further hydrogenated, resulting in an increased amount of stearic acid. The requirement of a free carboxyl group for hydrogenation establishes that lipolysis must precede BH (Hawke and Silcock, 1970).

After these initial studies demonstrated BH in ruminal contents, various in vivo and in vitro experiments were carried out to examine the BH pathways of unsaturated fatty acids from the diet. An in vivo experiment by Wood et al. (1963) provided further evidence for the BH pathway of linoleic acid. Four wethers received an injection of ^{14}C-labeled linoleic acid through the abdominal wall into the rumen after ligation of the reticulo-omasal orifice. After 48 h, they observed that 85 to 96% of the dose remained in the rumen, 46% was completely saturated, 33 to 50% was hydrogenated to oleic or elaidic acid, and 3 to 6% remained as linoleic acid. Ward et al. (1964) incubated whole ruminal contents in an artificial rumen with [U-^{14}C]-labeled linolenic, linoleic, and oleic acids. The linolenic acid was rapidly hydrogenated. There was conversion to 18:2, 18:1, and stearic acid, supporting the results of Shorland et al. (1955). They observed that 93% of the linoleic acid was converted to stearic acid. There was also an accumulation of a small amount of 18:1 that was predominantly *trans*. The oleic acid was rapidly converted to stearic acid, with only a small amount of the radioactivity in the *trans*-18:1 fraction.

Wilde and Dawson (1966) constructed a metabolic scheme for the BH of α-linolenic acid to stearic acid by incubating sheep ruminal contents with α-[U-^{14}C]-linolenic acid. The results of this in vitro experiment supported those of Ward et al. (1964). The initial step was the isomerization of the *cis*-12 bond to either the C_{11} or C_{13} position. Next, one of the bonds was hydrogenated to leave an 18:2, followed by hydrogenation of another bond, producing an 18:1. Hydrogenation of the 18:1 formed stearic acid as the final product.

Pure cultures of ruminal microbes have also been used to explore the different BH pathways. Kepler et al. (1966) examined the BH pathway of linoleic acid by using the bacterium *B. fibrisolvens*. The linoleic acid was initially isomerized to a conjugated 18:2, possibly *cis*-9, *trans*-11-CLA. The *cis*-9, *trans*-11-CLA was then hydrogenated, leaving a *trans*-18:1 as the final product. However, in mixed ruminal bacteria, the 18:1 was further hydrogenated to stearic acid (Ward et al., 1964). Kepler and Tove (1967) also incubated *B. fibrisolvens* with linoleic and linolenic acids. They confirmed that linoleic acid was first isomerized to *cis*-9, *trans*-11-CLA, followed by further hydrogenation to a mixture of *trans*-18:1. When linolenic acid was used as a substrate, it was first isomerized to *cis*-9, *trans*-11, *cis*-15-18:3. This acid was further hydrogenated to a nonconjugated 18:2 as the final product.

White et al. (1970) isolated a small gram-negative capsulated anaerobic bacillus and tested its ability to hydrogenate unsaturated fatty acids. Approximately 80% of the oleic acid added to the culture was converted to stearic acid within 72 h. Traces of *trans*-18:1 were also formed. When linoleic acid was used as a substrate,

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approximately 80% was converted to stearic acid within 72 h. Traces of labeled 18:1 and 18:2 were also present. When the substrate was linolenic acid, 80% was converted to a mixture of 18:1, and traces of a nonconjugated 18:2 were also present; however, stearic acid was not formed.

Kemp et al. (1975) isolated 5 anaerobic bacteria from sheep rumen that were able to hydrogenate unsaturated fatty acids. Two *Fusocillus* bacteria could hydrogenate oleic and linoleic acids to stearic acid and also could hydrogenate linolenic acid to cis-15-18:1. *Ruminococcus albus* and 2 *Eubacterium* did not hydrogenate oleic acid, but metabolized linoleic and linolenic acids to a mixture of 18:1. The intermediates and products of hydrogenation of these bacteria were the same as previously found in vitro and in vivo with mixed ruminal microbes.

MICROORGANISMS INVOLVED IN BH

The rumen microbial population consists mainly of ciliate protozoa, anaerobic bacteria, and anaerobic fungi. The bacteria are genetically very diverse, deriving from many different origins (Tajima et al., 1999, 2000, Edwards et al., 2004), whereas the protozoa are monophyletic, thought to have evolved from a symbiotic protozoan that established in the rumen 30 to 40 million years ago (Wright et al., 1997). The fungi have several morphotypes, but these are very closely related to each other (Li and Heath, 1992). This mixture of organisms digests the food that the animal consumes, and the products of microbial digestion form the majority of nutrients that sustain the ruminant animal itself. The role of the different microbial species in the BH of unsaturated fatty acids was reviewed in 1997 by Harfoot and Hazlewood (1997), who noted that little had changed in the previous decade since their previous review in 1988. Significantly, virtually all the information predated the discovery of the importance of CLA (Ha et al., 1987) and also of modern microbial genetics and molecular phylogenetic techniques for identifying and classifying microorganisms by their small-subunit rRNA gene sequences (Edwards et al., 2004; Eckburg et al., 2005). These factors have provided new information about BH since research interest was rekindled by increasing concerns about fatty acids and human health.

Role of Bacteria

Early microbiological studies (Polan et al., 1964; Kemp et al., 1975; Hazlewood et al., 1976) were carried out before the significance to health of n-3 PUFA, CLA, and *trans*-11-18:1 were known. *Butyrivibrio fibrisolvens* was identified as undertaking BH of fatty acids and forming CLA and *trans*-11-18:1 as intermediates in the process of BH of linoleic acid (Polan et al., 1964; Kepler et al., 1966). Stearic acid was not formed from linoleic acid by *B. fibrisolvens,* however. Later studies (Kemp et al., 1975; Hazlewood et al., 1976) identified other bacteria that were capable of BH, but they did not provide much information about relative activities. Methods in these studies used radiolabeled substrates and were highly sensitive, but the concentrations of labeled acids used as substrates were very low (2 μg/mL), making comparison of the activity and quantitative significance of different bacteria difficult. Most isolates converted linoleic acid only as far as 18:1, mainly *trans*-11-18:1; these were termed group A by Kemp et al. (1975). Bacteria carrying out stearate formation (group B) were identified as *Fusocillus* spp. (Kemp et al., 1975), a genus description that, like the cultures themselves, has not survived. Cultures deposited in culture collections proved to be nonviable; thus, the bacteria have not been subjected to modern molecular phylogenetic analysis. More recently, van de Vossenberg and Joblin (2003) isolated from a grazing cow a bacterium that could also form stearate from linoleate. It was phenotypically similar to *Fusocillus,* and their analysis indicated that it was phylogenetically close to *Butyrivibrio hungatei.*

Recent investigations at the Rowett Research Institute have attempted to evaluate the quantitative significance of different bacterial species in BH, and to identify the most crucial bacteria for the conversion of *trans*-11-18:1 to stearic acid. First, the properties of different bacteria from culture collections were assessed (Maia et al., 2006). The concentrations of fatty acids used were high enough to reveal quantitative patterns not only of metabolism, but also of toxicity, which is relevant to microbial ecology and nutrition. Eleven of 26 predominant species of ruminal bacteria metabolized linoleic acid substantially, apparently by several possible routes. The most common product was *trans*-11-18:1, produced by 3 *Butyrivibrio* strains and 2 strains of *Clostridium proteoclasticum.* Only *C. proteoclasticum* produced 18:0. Because *trans*-11-18:1 is formed via *cis*-9, *trans*-11-CLA as an intermediate, bacteria forming *trans*-11-18:1 might also be considered *cis*-9, *trans*-11-CLA producers. In spite of its name, *C. proteoclasticum* is related closely to *Butyrivibrio* (Attwood et al., 1996; Kopecny et al., 2003; Paillard et al., 2007). The conclusion that *Butyrivibrio* is of principal importance in ruminal BH is similar to the original results of Polan et al. (1964), who investigated more than 20 strains of ruminal bacteria. A related, as yet only partly published study (Wallace et al., 2006) screened fresh isolates from the sheep rumen. Four hundred random isolates were screened for their ability to metabolize linoleic acid. All bacteria that produced substantial quantities of *cis*-9, *trans*-11-CLA, *trans*-11-18:1, or both from linoleic acid were butyrate producers. Not all butyrate producers formed *cis*-9, *trans*-11-CLA or *trans*-11-18:1, however. Only bacteria falling within the *B. fibrisolvens* group formed *cis*-9, *trans*-11-CLA or *trans*-11-18:1. Butyrate-producing *Eubacterium* and *Clostridium* spp. did not. A few of the bacteria that formed CLA, *trans*-11-18:1, or both also formed stearic acid. The stearate producers were similar in morphol-

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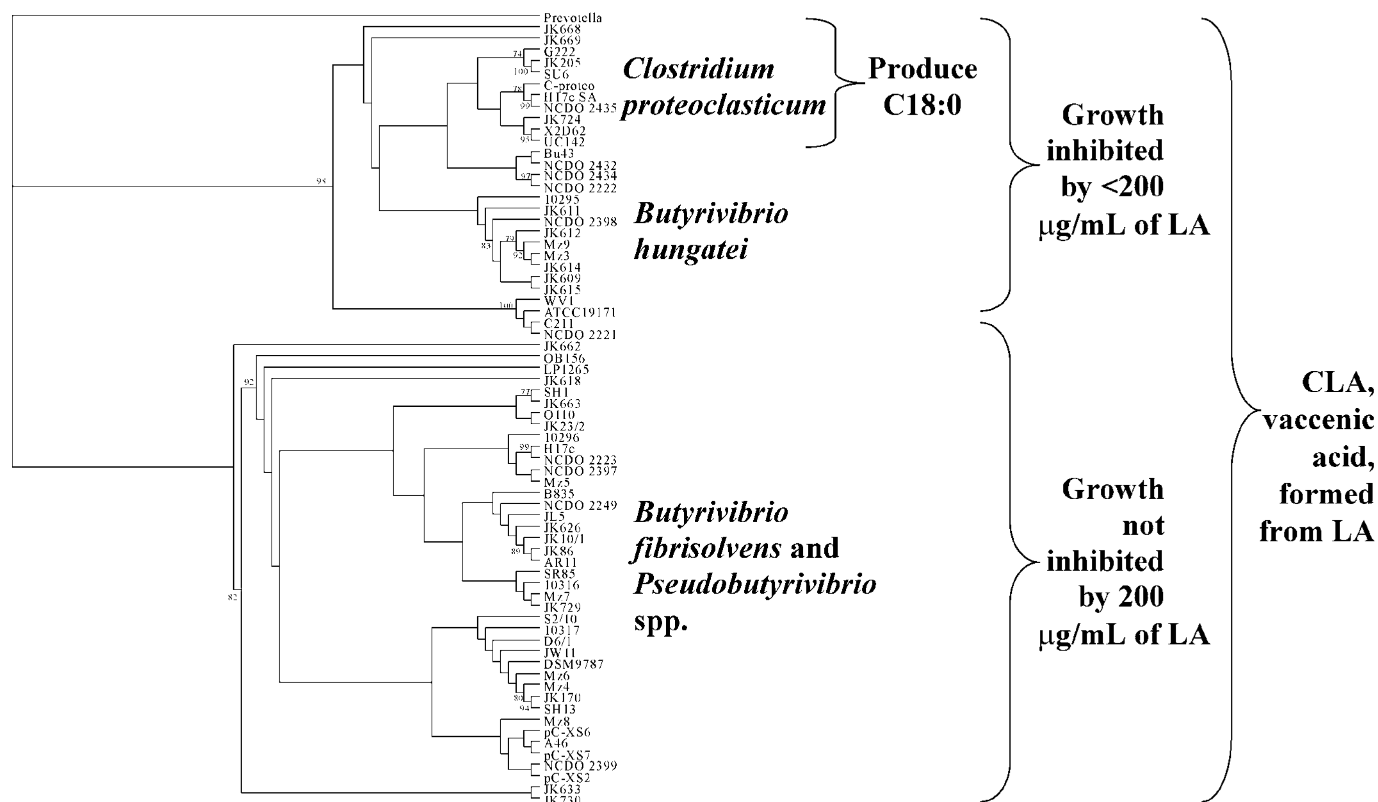

Figure 2. Phylogenetic tree based on 16S rRNA sequence analysis of ruminal bacteria related to *Butyrivibrio,* their metabolism of linoleic acid (LA), and their sensitivity to growth inhibition by LA.

ogy and metabolic properties to *Fusocillus* spp. isolated many years ago (Kemp et al., 1975). Phylogenetic analysis based on 16S rRNA sequence analysis (Figure 2) indicated that the stearate producers clustered on a branch with *C. proteoclasticum,* which was also found to form stearate. The identification of strain Su6 as *B. hungatei* (van de Vossenberg and Joblin, 2003) was incorrect, being based on a much smaller number of 16S rDNA sequences than the 69 used by Paillard et al. (2007). Stearate-producing strain Su6 clusters most closely with *C. proteoclasticum.* Given the importance of this bacterium to the BH process, and its irreversible removal of health-promoting fatty acids, the suppression of *C. proteoclasticum* must be regarded as a significant health issue for humans.

Role of Ciliate Protozoa

Up to half of the rumen microbial biomass may be protozoal (Williams and Coleman, 1992) and approximately three-quarters of the microbial fatty acids present in the rumen may be present in protozoa (Keeney, 1970). It has been known for many years that protozoal lipids contain proportionally more unsaturated fatty acids than the bacterial fraction (Katz and Keeney, 1966; Harfoot and Hazlewood, 1997). Thus, protozoa could represent a very important source of PUFA, CLA, and *trans*-11-18:1 for incorporation into meat and milk. Wright (1959, 1960) concluded that both protozoa and bacteria were involved in BH, but the extensive ingestion of bacteria by protozoa was considered by others (Dawson and Kemp, 1969) to cast doubt on this conclusion. Biohydrogenation in ruminal digesta was only slightly decreased following defaunation, and the presence of protozoa was not necessary for BH to occur (Dawson and Kemp, 1969). Girard and Hawke (1978) and Singh and Hawke (1979) also suggested that the minor contribution of protozoa to the BH process was due to the activity of ingested or associated bacteria.

Recently, Devillard et al. (2006) revisited the issue of whether protozoa form unsaturated fatty acids, with a focus on CLA and *trans*-11-18:1. As found earlier by others (Katz and Keeney, 1966; Viviani, 1970; Emmanuel, 1974), mixed protozoa from the sheep rumen contained at least 2 to 3 times more unsaturated fatty acids than bacteria. These unsaturated fatty acids included CLA and *trans*-11-18:1, which were more than 8- and 3-fold more abundant in protozoa than in bacteria, respectively (Figure 3). Different species had different compositions, with larger species, including *Ophryoscolex caudatus,* containing more than 10 times higher concentrations of CLA and *trans*-11-18:1 than some small species, such as *Entodinium nannelum. Isotricha prostoma,* a large species and the only holotrich examined, had low concentrations of CLA and *trans*-11-18:1. In incubations with fractionated ruminal digesta, linoleic acid metabolism was very similar in strained ruminal fluid and its derived bacterial fraction, whereas its

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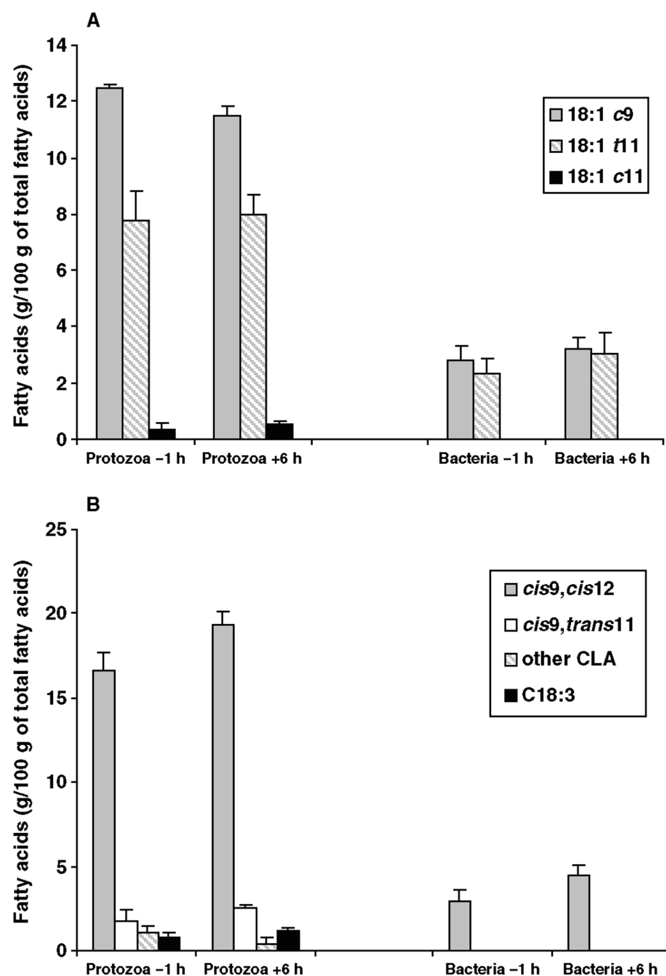

Figure 3. Composition of the main unsaturated C^{18} fatty acids of mixed ruminal protozoa and bacteria isolated from the rumen of sheep 1 h before feeding and 6 h after feeding. (A) Monounsaturated C^{18} fatty acids; (B) polyunsaturated C^{18} fatty acids. Values are means ± SE for 4 sheep. From Devillard et al. (2006), with permission.

mixed protozoal fraction had much lower activity. The opposite direction of reaction, namely desaturation, also did not occur in the protozoal fraction. Radioactivity from ^{14}C-stearate was not incorporated into CLA or *trans*-11-18:1 by protozoa. No genes with sequence similarity to fatty acid desaturase genes from other organisms were found in cDNA libraries from ruminal protozoa (E. Devillard, Rowett Research Institute, Bucksburn, Aberdeen, UK, unpublished data). Thus, the protozoa are rich in CLA and *trans*-11-18:1, yet they appear not to synthesize these 2 fatty acids from stearate.

It has been argued that the high unsaturated fatty acid content of protozoa results from the ingestion of plant particles, especially chloroplasts (Wright, 1959; Stern et al., 1977). This cannot explain the high concentration of CLA and *trans*-11-18:1 in protozoa, however, because these fatty acids are absent from the plant material. Bacterial activity must be involved. The most likely explanation is that protozoa preferentially incorporate CLA and *trans*-11-18:1 formed by ingested bacteria. In our experience, the relative activities of the individual steps of the pathway outlined in Figure 1 are isomerase << CLA reductase < *trans*-11-18:1 reductase. Thus, as protozoa digest the protein of ingested bacteria, the last step will decline to very low levels while there is still substantial activity at the other steps, resulting in the formation of CLA and linoleic acid being maintained, but with a greatly decreased further metabolism to stearic acid.

These findings have implications for the role of protozoa in the flow of CLA and *trans*-11-18:1 from the rumen. The availability of these fatty acids for absorption by the host animal could depend on the flow of protozoa rather than on bacteria from the rumen. Some ciliate protozoa are retained selectively within the rumen by a migration or sequestration mechanism that depends on chemotaxis (Abe et al., 1981; Ankrah et al., 1990). As a consequence, protozoal biomass reaching the duodenum is proportionally less than would be expected if they were to flow with the rest of ruminal digesta (Weller and Pilgrim, 1974). It might be imagined that this selective retention would be detrimental to the flow of CLA and *trans*-11-18:1 into meat and milk. The flow of microbial N at the duodenum of steers was recently shown to be 12 to 15% protozoal in origin, whereas in terms of fatty acid flow, protozoa accounted for between 30 and 43% of the CLA and 40% of the *trans*-11-18:1 reaching the duodenum (Yanez-Ruiz et al., 2006). The contribution of protozoa to the flows of 16:0 and 18:0 to the duodenum was less than 20 and 10%, respectively. Thus, protozoa do not themselves produce CLA and *trans*-11-18:1 by their own metabolism; nevertheless, they might be expected to have a significant influence on CLA and *trans*-11-18:1 available to the host animal. However, the situation about PUFA flow in general remains unclear. In defaunated sheep, the concentration of SFA in the blood remained the same as in conventional sheep, whereas the concentration of 18:2 and 18:3 tended to increase when the protozoa were removed (Klopfenstein et al., 1966; Abaza et al., 1975).

Role of Anaerobic Fungi

Anaerobic fungi form a minor part of the biomass of ruminal microorganisms, but they may contribute significantly to overall metabolism, as is the case with their high cellulolytic activity (Wilson and Wood, 1992). Ruminal fungi have been known for some time to contain a relatively high 18:1 composition (Kemp et al.,1984b; Body and Bauchop, 1985), and evidence from labeling experiments indicated that Δ^9-desaturase converted stearic acid to 18:1n-9 fatty acids in *Piromyces communis* (Kemp et al., 1984b). Linoleic acid and linolenic acid incubated with *P. communis* resulted in the formation of conjugated products (Kemp et al., 1984b). Recent work by Nam and Garnsworthy (2007) con-

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firmed that the major product of linoleic acid metabolism was *cis*-9, *trans*-11-CLA.

In a recent study (Maia et al., 2006), 2 species of ruminal fungi, *Neocallimastix frontalis* and *P. communis,* were grown in medium to which 50 μg/mL linoleic acid had been added. At 96 h following a 5% inoculum, *N. frontalis* had grown in the linoleic acid-containing medium, whereas no growth was observed with *P. communis* in the presence of linoleic acid or other PUFA at the same concentration. *Neocallimastix frontalis* had metabolized approximately half of the linoleic acid in the medium, forming *cis*-9, *trans*-11-CLA. This activity of *N. frontalis* in forming CLA from linoleic acid is very small in comparison with *B. fibrisolvens;* a similar conversion of 50 μg/mL by a culture of the ruminal bacterium, *B. fibrisolvens,* is achieved in a few minutes.

INTERMEDIATES OF BH

One area that has received renewed attention is attempting to establish the pathways of BH and the identity of intermediates. In vivo studies have revealed a vast array of *trans*-18:1 and CLA isomers present in the digesta contents of cattle and sheep. These in vivo studies have been important in showing that many more intermediates exist in ruminal contents than can be accounted for by most accepted pathways of BH. However, it is difficult or impossible to determine the origin of a specific BH intermediate in ruminal contents when the diet contains a multitude of fatty acids.

Many of the advances in determining the origin of BH intermediates were accomplished from pure culture studies in which the media contained a single fatty acid in the substrate. An alternative approach has been to isotopically label one or more carbons in a fatty acid substrate and then trace labeled carbon transfer to intermediates. In earlier studies on BH, this was done with radioactive isotopes, which have been replaced with stable isotopes in more recent times. The problem with isotopic tracer studies is that they reliably identify whether an intermediate arises from a specifically labeled compound, but do not reveal the number or identity of intermediate steps. Stable isotopes of intermediates must then be incubated with ruminal microorganisms in subsequent studies to build the entire metabolic pathway.

The remainder of this section summarizes recent advances that have been made in documenting the origin of one or more *trans*-18:1 and CLA intermediates arising from the BH of specific unsaturated fatty acids. Considerable progress has been made in tracing intermediates of oleic and linoleic acid BH. Less is known about the identity of intermediates arising from BH of the n-3 fatty acids, including linolenic acid and the major fish oil fatty acids.

Oleic Acid

Oleic acid BH is often shown as proceeding directly to stearic acid without the formation of intermediates (Kellens et al., 1986). However, *cis/trans* isomerization is common among bacterial species and has been demonstrated in a limited number of ruminal bacteria. Bacterial species acquired the capability of *cis* to *trans* isomerization to alter plasma membrane permeability as a protection device against growth inhibitors or environmental insults (Okuyama et al., 1991). A nonruminal bacteria, *Pseudomonas* strain NRRL, can transform oleic acid to *trans*-10-18:1, but only under low-pH growth conditions (Mortimer and Niehaus, 1972). When the capacity for *cis/trans* isomerization was examined in several bacterial species from the ruminal contents of sheep, only *Fusocillus* T344 was capable of converting oleic acid to *trans*-11-18:1 (Kemp et al., 1975). Thus, production of *trans*-18:1 from oleic acid in the rumen likely depends on specific environmental conditions in ruminal contents that favor *cis/trans* isomerization.

Oleic acid conversion to *trans*-18:1 was recently reevaluated (Mosley et al., 2002; Proell et al., 2002; AbuGhazaleh et al., 2005) by using ^{13}C-labeled oleic acid and *trans*-9-18:1 in ruminal batch cultures. In batch rumen cultures, incubation of oleic-1-^{13}C acid (*cis*-9 18:1) resulted in ^{13}C detection in stearic acid as well as multiple *trans*-18:1 isomers having double bond positions from C_6 to C_{16} (Mosley et al., 2002, 2006). Furthermore, the addition of ^{13}C-labeled *trans*-9-18:1 demonstrated the isomerization of the *trans*-9 bond to other *cis*- and *trans*-18:1 isomers (Proell et al., 2002). These experiments provided further evidence of *cis/trans* isomerization of oleic acid by mixed ruminal microorganisms, yielding a wide range of positional *trans*-18:1 isomers. Additional studies demonstrated that oleic acid isomerization could be dramatically altered by environmental conditions in the rumen. In continuous flow ruminal fermenters, isomerization of oleic-1-^{13}C acid to *trans*-18:1 was restricted to double bond positions $<C_{10}$ with decreasing pH (6.5 to 5.5) and decreasing dilution rate (0.10 to 0.05/h; AbuGhazaleh et al., 2005).

Conversion of *trans*-18:1 to stearic acid is variable. Early work by Kemp et al. (1984a) tested the ability of *Fusocillus* sp. to hydrogenate *cis* and *trans* 18:1 to stearic acid. *Cis*-5 to *cis*-13 and *trans*-5 to *trans*-13 isomers were all hydrogenated to some extent by late log-phase cultures incubated for 3 h. Between 73 and 79% of *cis*-5 to *cis*-11 isomers were converted to stearic acid. However, *cis*-12 (30%) and *cis*-13 (5%) were poorly hydrogenated. Of the *trans* isomers, 45% of *trans*-8, *trans*-9, and *trans*-10 were converted to stearic acid, but other isomers were poorly hydrogenated. *Cis*-2, *cis*-4, and *cis*-5 (90%) were converted to stearic acid. *Cis*-6 to *cis*-12 and *trans*-8 to *trans*-10 (75%) were converted to stearic acid.

Other documented intermediates of oleic acid BH, aside from *trans*-18:1 isomers, include derivatives of stearic acid. Several studies (Hudson et al., 1995; Morvan and Joblin,1999; Jenkins et al., 2006) reported the conversion of oleic acid to hydroxystearic and ketostearic acids. Production of hydroxy and ketostearic

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Table 1. References that have identified conjugated linoleic acid isomers present in ruminal contents according to position and geometry of double bonds[1]

Carbon positions	*cis/cis*	*cis/trans*	*trans/cis*	*trans/trans*
7,9			a	ab
8,10			ae	abd
9,11	cde	abcd	e	abd
10,12		e	abcde	abd
11,13	d	abde	abe	abcde
12,14		b		

[1]a = Piperova et al. (2002); b = Shingfield et al. (2003); c = Loor et al. (2002); d = Duckett et al. (2002); e = Loor et al. (2004).

acids accounted for 6 to 10% of oleic acid net loss in rumen batch cultures, and up to 30% of oleic acid net loss in continuous cultures of mixed ruminal microorganisms (Jenkins et al., 2006).

Some direct conversion of oleic acid to stearic acid has not been ruled out, but it is clear that oleic acid can be transformed to a variety of lipid compounds other than just stearic acid. Jenkins et al. (2006) proposed a pathway for transformation of oleic acid by ruminal microorganisms that included its direct hydrogenation to stearic acid, but also included its isomerization to 18:1 isomers having double bond positions from C_6 through C_{16}, and its hydration to 10-hydroxystearic acid. The proposed pathway also suggested oxidation of hydroxystearic acid to ketostearic acid, and some *trans* to *cis* isomerization of elaidic acid, leading to limited synthesis of oleic acid.

Linoleic Acid

Despite past evidence that ruminal BH is a complex biochemical process involving a wide range of fatty acid intermediates, the published pathways for linoleic acid BH have changed very little over the decades. For example, the conversion of linoleic acid to stearic acid included only 2 intermediates (*cis*-9, *trans*-11-CLA and *trans*-11-18:1) in the pathway proposed by Garton (1977). This pathway persisted over time and is often depicted in an identical manner in current publications (Ribeiro et al., 2007). A vast array of *trans*-18:1 and CLA isomers have now been identified in digesta contents from cattle. In vivo and in vitro studies reported CLA isomers having double bond positions that range from $C_{7,9}$ to $C_{12,14}$ (Table 1). Accounting for all geometric isomers reported, a total of 14 CLA isomers were identified in digesta contents from cattle or in vitro contents. Total in vivo production of CLA isomers averages approximately 10 g/d for *cis-trans* CLA classes and 5 g/d for *trans-trans* CLA classes (Teter and Jenkins, 2006). In most cases, *cis*-9, *trans*-11-CLA is the most prevalent isomer found in ruminal contents of cattle and probably was the only isomer that could be detected in many older chromatographic techniques. Thus, *cis*-9, *trans*-11-CLA persisted over time as the only CLA isomer seen in pathways of BH. The availability of longer columns (100 m or more) for GLC combined with new techniques in mass spectroscopy have enabled separation and identification of minor CLA isomers.

Aside from *cis*-9, *trans*-11-CLA, another CLA isomer reported to arise from linoleic acid BH is *trans*-10, *cis*-12-CLA. Griinari and Bauman (1999) proposed that *cis*-9, *trans*-11-CLA originated from linoleic acid, with it eventual conversion to *trans*-10-18:1 in ruminal contents. Although Griinari and Bauman (1999) only speculated on the production of *trans*-10, *cis*-12-CLA, later work by Kim et al. (2002) demonstrated significant production of *trans*-10, *cis*-12-CLA by some *Megasphaera elsdenii* strains. *Bifidobacterium, Propionibacterium, Lactococcus, Streptococcus,* and *Lactobacillus* isolates from other habitats have also been shown to form *trans*-10, *cis*-12-CLA (Jiang et al., 1996; Coakley et al., 2003; Ando et al., 2004). Because these genera occur in the rumen, although generally at rather low numbers, they may contribute to BH, and specifically to *trans*-10, *cis*-12-CLA formation in the rumen. *Propionibacterium, Streptococcus,* and *Lactobacillus* are also more numerous in the rumen with concentrate diets (Stewart et al., 1997), which would again be consistent with greater *trans*-10, *cis*-12-CLA production with concentrate diets.

Very few other BH intermediates from linoleic acid have been documented. *Butyrivibrio fibrisolvens* produced *trans*-9, *trans*-11-CLA from linoleic acid at concentrations of approximately one-tenth of *cis*-9, *trans*-11-CLA (Wallace et al. 2007). In addition, *Bifidobacterium breve* species isolated from human intestinal contents converted linoleic acid to *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA isomers, which were then isomerized to *trans*-9, *trans*-11-CLA under anaerobic growth conditions. Ogawa et al. (2001) reported the formation of 10-hydroxy *cis*-12-18:1 from linoleic acid by *Lactobacillus acidophilus,* which they speculated might be the initial intermediate in linoleic acid BH that is subsequently oxidized to *cis*-9, *trans*-11-CLA.

A recent study completed at Clemson University incubated 1-^{13}C-linoleic acid with mixed ruminal microorganisms in batch culture to determine the identity of CLA isomers formed from linoleic acid. Enrichment of ^{13}C and structural identity of the double bond position and geometry were determined by chemical ionization gas chromatography-mass spectroscopy. Seven CLA isomers were enriched, indicating their formation from linoleic acid. All enriched CLA isomers had double bonds in either the 9,11 or 10,12 positions. Within each class of positional isomer (i.e., 9,11 or 10,12), early results indicated that all possible *cis-trans* combinations were represented except for *trans*-9, *cis*-11.

n-3 Fatty Acids

According to most accounts of BH (Figure 1), linolenic acid is transformed initially to *cis*-9, *trans*-11, *cis*-15-18:3 by ruminal microorganisms, which is subsequently hydrogenated to the nonconjugated *trans*-11, *cis*-15

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18:2. If correct, linolenic acid BH yields a conjugated double bond in its initial isomerization step, but does not lead to the formation of any CLA isomers. Determining whether linolenic acid and other n-3 fatty acids can yield CLA upon BH is of particular importance because of the wide range of metabolic and physiological effects reported for various CLA isomers. The eventual goal would be to regulate BH to the point that any number of desired CLA isomers could be delivered to body tissues to achieve a desired physiological outcome.

The desire to focus on major intermediates and avoid complication of BH has led to the persistence of many oversimplified pathways over the decades. Additional intermediates of linolenic acid BH have been recognized for many years. For instance, Dawson and Kemp (1969) stated that "incubation of [^{14}C]linolenic acid with rumen microorganisms resulted in a bewildering number of radioactive C_{18} acids with various degrees of unsaturation and positional isomerization." Regardless, very few studies have analyzed 18:3 isomers present in the ruminal contents of cattle, making it difficult to expand the pathways of linolenic acid BH with any degree of confidence.

Loor et al. (2004) reported duodenal flow of 3 18:3 isomers (*cis*-9, *trans*-12, *cis*-15-18:3; *cis*-9, *trans*-12, *trans*-15-18:3; *trans*-9, *trans*-12, *trans*-15-18:3) in dairy cows fed conventional diets. Feeding linseed oil to cows in their study increased the duodenal flow of all 3 isomers. It was implied that these 3 18:3 isomers originated from BH of linolenic acid, assuming that 18:3 intermediates could arise only from parent fatty acid compounds with 3 or more double bonds.

Destaillats et al. (2005) reported that 2 conjugated 18:3 isomers were present in milk fat at low concentrations (0.3%), namely, the *cis*-9, trans-11, *cis*-15-18:3 and the *cis*-9, *trans*-13, *cis*-15-18:3 isomers. From their appearance in milk fat, Destaillats et al. (2005) proposed both isomers as the initial intermediates of linolenic acid BH, which are then subsequently reduced to 2 nonconjugated dienes (*trans*-11, *cis*-15-18:2; *cis*-9, *trans*-13-18:2) and 2 conjugated dienes (*cis*-9, *trans*-11-CLA; *trans*-13, *cis*-15-CLA). Waşowska et al. (2006) presented an alternative view of linolenic acid BH. Linolenic acid BH in strained rumen fluid led to the accumulation of 2 18:3 isomers (*cis*-9, *trans*-11, *cis*-15-18:3; *trans*-9, *trans*-11, *cis*-15-18:3) and 1 nonconjugated 18:2 isomer (*trans*-11, *cis*-15-18:2), but accumulation of *cis*-9, *trans*-11-CLA was not seen.

Intermediates formed from BH of fatty acids having more than 3 double bonds (such as 20:5 and 22:6 in fish oil) are even less clear. Both 20:5 and 22:6 disappear over time when incubated in cultures of mixed ruminal microorganisms, which AbuGhazaleh and Jenkins (2004) proposed could be due to isomerization, hydrogenation, or chain shortening. If consistent with pathways for linolenic acid, the initial isomerization of 20:5 and 22:6 would be expected to produce isomers with 5 and 6 double bonds, with at least 1 double bond having *trans* geometry. Isomerization would be followed by hydrogenation to isomers with 4 and 5 double bonds. However, none of these have been clearly identified in digesta contents of cattle fed fish oil.

ENZYMIC MECHANISMS OF BH

Previous work identified BH as a 2-step process involving the sequential action of 2 classes of microbial enzymes, isomerases and reductases. Kepler and Tove (1967) examined the initial isomerization reaction of linoleic acid, in which the *cis*-12 bond is converted to a *trans*-11 bond, in the presence of *B. fibrisolvens*. This reaction occurs away from any functional groups and does not require a cofactor. The maximum activity of the isomerase enzyme was found to be within a narrow concentration range of substrate, greater than 5×10^{-5} *M* for linoleic acid and greater than 10^{-4} *M* for linolenic acid. Kepler et al. (1970) also examined the linoleate isomerase enzyme of *B. fibrisolvens*. They concluded that 3 features of the substrate are involved in the binding of the substrate to the enzyme. These properties are (1) the π system of a substrate double bond, (2) hydrophobic interaction, and (3) hydrogen bonding of the substrate carboxyl group. Kepler et al. (1971) found that the isomerase enzyme was highly specific for *cis*-9, *cis*-12-CLA, with the final double bond 6 carbons from the terminal methyl group. They demonstrated that there was stereospecific addition of hydrogen to C_{13} in the D configuration. It was proposed that when the fatty acid binds to the hydrophobic pocket of the enzyme, it is forced into a loop. The initial binding involves the interaction of the π-electrons of the substrate double bond with an electrophilic group on the enzyme. The carboxyl group of the substrate is then bound to the electronegative center on the enzyme via hydrogen bonding. After the enzyme-catalyzed isomerization is complete, the product is then released.

The second reaction of the BH of linoleic acid by *B. fibrisolvens* is the hydrogenation of *cis*-9, *trans*-11-CLA to *trans*-11-18:1. Rosenfeld and Tove (1971) examined the source of hydrogen and the stereospecificity of the reduction of the double bond with intact cell cultures. Their experiments showed that deuterium was incorporated at carbons having a *cis* double bond prior to reduction. Rosenfeld and Tove (1971) concluded that this reduction reaction was stereospecific and speculated that the reduction occurs by addition of a proton and hydride ion mediated by an unknown carrier. Hunter et al. (1976) used cell-free preparations of *B. fibrisolvens* to demonstrate that reduced methyl viologen, NADH, and an endogenous electron donor could each serve as a reductant. Their results indicated the presence of at least 2 hydrogenation systems and the possible involvement of a flavoprotein. They also observed that the isomerase and reductase enzymes of *B. fibrisolvens* were separable by gel filtration and did not appear to be part of a multienzyme complex; however, the enzymes did appear to be a part of the cell membrane.

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Although much was learned about the formation of *cis*-9, *trans*-11-CLA from Tove and his group, it seemed impossible to purify the isomerase enzyme to homogeneity and retain its activity (Kepler and Tove, 1967). Moreover, the kinetics of the enzyme in cell extracts were strange, with the reaction not proceeding to the equilibrium position of a K_s of 61 in favor of CLA and the enzyme not recycling in a way that would be expected of an isomerase (Kepler and Tove, 1967; Kim et al., 2000). In contrast, the linoleate isomerase gene from *Propionibacterium acnes* has been overexpressed and the enzyme has been crystallized in 6 different molecular forms (Liavonchanka et al., 2006). As a result, the precise conformation of substrate, AA residues, and cofactor (FAD) at the active site of the enzyme has been established, and the exact stereochemistry of the isomerization can be drawn in molecular models.

There has been considerable recent interest in the CLA isomerase, with the prospect of producing CLA by biotechnological means. For example, Fukuda et al. (2006) isolated a strain of *B. fibrisolvens* that accumulates *cis*-9, *trans*-11-CLA because it lacks the ability to hydrogenate it to *trans*-11-18:1. Ultimately, it will be necessary to understand the enzymes, their genes, and the mechanism of BH to fulfill the commercial possibilities of biotechnological CLA production from linoleic acid.

The situation with the linoleate isomerase that forms *cis*-9, *trans*-11-CLA is much different, but there has been some recent progress in experiments in which samples of digesta from the sheep rumen and individual species of ruminal bacteria were incubated with linoleic acid in buffer containing deuterium oxide (Wallace et al., 2007). Mass spectrometric analysis indicated that *cis*-9, *trans*-11-CLA and *trans*-9, *trans*-11-CLA formed in mixed digesta were labeled with 1 atom of deuterium at C_{13}, whereas *trans*-10, *cis*-12-CLA was unlabeled. The same was true in pure cultures of *B. fibrisolvens* and *C. proteoclasticum.* Thus, the mechanism of formation of *trans*-10, *cis*-12-CLA is well known and is typical of a genuine isomerase, whereas the formation of *cis*-9, *trans*-11-CLA is not. It has been suggested that the synthesis of 9,11 isomers may be initiated by hydrogen abstraction on C_{11} catalyzed by a radical-intermediate enzyme (Wallace et al., 2007). Given the biotechnological implications of being able to produce bulk quantities of pure *cis*-9, *trans*-11-CLA, it will be important to explore this possible mechanism in greater detail.

An interesting question can be raised about why bacteria developed the enzymatic capacity to carry out BH. The question is not purely academic, because the mechanism may hold the key to finding methods to manipulate BH in a predictable manner. Two main explanations have been proposed. Lennarz (1966) suggested that BH was a means of disposing of reducing power, whereas Kemp and Lander (1984) and Kemp et al. (1984a) proposed that BH was in fact a detoxification mechanism.

The reducing power disposal hypothesis seems difficult to sustain. The disposal of hydrogen in an anaerobic ecosystem is vital to fermentation. However, the amount of hydrogen equivalents that would be removed by BH of unsaturated fatty acids is small in proportion to total metabolism. For example, Ulyatt et al. (2002) measured 431 g of methane produced daily from dairy cows consuming 19.3 kg of DM/d of grass with a lipid content of 4.4%. Methane production was therefore 26.9 (431/16) mol/d, and because it consumed 4 mol of H_2 per mole of methane, the hydrogen utilized for methane production would be $4 \times 26.9 = 108$ mol/d. The lipid might contain approximately 50% linolenic acid, resulting in an estimated linolenic acid daily intake of $0.425\,(0.5 \times 0.044 \times 19.3)$ kg = 1.53 (425/278) mol, requiring daily $3 \times 1.53 = 4.59$ mol of H_2 for saturation to 18:0.

It might be argued that any additional advantage gained by one species, however slight, may provide a crucial edge in a highly competitive microbial ecosystem. Yet the reductase that converts CLA to *trans*-11-18:1 in *B. fibrisolvens* constitutes 0.5% of the total cell protein (Hughes et al., 1982), which in itself represents a very significant expenditure of cellular resources (unless the enzyme has another function as well, which remains to be proved). Even more convincing was the argument made by Harfoot and Hazlewood (1997) that *B. fibrisolvens* possesses a hydrogenase, which is a much more efficient method for removing excess reducing power than biohydrogenating unsaturated fatty acids.

The detoxification hypothesis seems to carry more weight. The sensitivity of different isolates in the *Butyrivibrio*-related phylogenetic tree was highly variable, with the *C. proteoclasticum* group being much more sensitive than other *B. fibrisolvens* isolates (Paillard et al., 2007). Indeed, *C. proteoclasticum* was also sensitive to *trans*-11-18:1, explaining why this group forms stearic acid; that is, the bacteria have to remove *trans*-11-18:1 to grow, whereas the other *Butyrivibrio* isolates can grow in the presence of *trans*-11-18:1, but not CLA. Linolenic acid was more toxic to *B. fibrisolvens* than linoleic acid or CLA (Maia et al., 2006), reflecting an extra double bond having to be metabolized. Thus, unsaturated fatty acids prevent growth, and BH reverses that inhibition. One fascinating observation is that the sensitivity of different strains in the *Butyrivibrio* phylogenetic tree to linoleic acid is correlated with their enzymic mechanism of butyrate formation, with those bacteria forming butyrate via butyrate kinase being much more sensitive than those strains producing butyrate via the acyl transferase mechanism (Paillard et al., 2007)

MODELING LIPOLYSIS AND BH

During the last 50 yr, there has been a disappointingly limited amount of work into the development and use of mathematical models to describe ruminal lipolysis and BH. In many of the early in vivo and in vitro

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investigations of lipolysis and BH, and even in the majority of recent investigations, mathematical modeling has consisted of little more than a comparison of concentrations of various fatty acids at defined incubation times of a test feed in rumen fluid (Hawke and Silcock, 1970; Noble et al., 1974; Harfoot et al., 1975).

To understand and model a complex system that encompasses ruminal lipolysis of dietary fats and the pathways of ruminal BH of unsaturated fatty acids, we must first break the system into its constituent parts. One major problem with this area of research is that researchers have often focused on a specific research question and therefore have not presented their data with a view toward its possible use in modeling the lipolysis-BH system. Thus, researchers have frequently not attempted to quantify the rates of lipolysis separately from those of BH. Instead, they have often presented rate constants that describe the "net" BH of certain unsaturated fatty acids, and their rate constants really describe an aggregation of lipolysis, isomerization, and hydrogenation processes (Beam et al., 2000; Enjalbert et al., 2003; Troegeler-Meynadier et al., 2003; Ribeiro et al., 2007).

A second issue related to the modeling of ruminal lipolysis and BH is that there does not appear to have been a systematic attempt to identify the specific nature of the kinetic processes involved. Instead, researchers have generally simply assumed that either zero-order or first-order kinetics applies, and this has influenced how they have presented their results. For example, Gerson et al. (1985) reported zero-order rate constants for lipolysis. Palmquist and Kinsey (1994) carried out in vitro studies and reported that when fat concentrations in incubation media were above 2.5 g/L, lipolysis occurred as a zero-order process. Fat concentrations of 2.5 g/L in rumen fluid in vivo are midrange and vary across diets and fat-feeding practices. However, Palmquist and Kinsey (1994) did not comment on the nature of lipolysis kinetics when fat concentrations were below 2.5 g/L. In contrast, Beam et al. (2000) reported first-order rate constants for lipolysis, but indicated that their rate constants declined when the amount of fat in the incubation medium was increased. Similarly, with respect to modeling BH, most researchers have assumed first-order kinetics (Harvatine and Allen, 2006; Ribeiro et al., 2007).

However, even as long ago as the 1970s and 1980s, there was evidence that lipolysis and BH are not simple zero-order or first-order processes. Noble et al. (1974) presented data that suggested reduced first-order rates of lipolysis (%/h) as the amount of trilinolein in the incubation medium increased. Furthermore, the data of Noble et al. (1974) suggested the existence of complex changes in the nature of the kinetics of BH as the amount of free linoleic acid in the incubation medium was increased. Further, Van Nevel and Demeyer (1996) reported that rates of lipolysis were reduced when rumen fluid pH declined below 6.0. Gerson et al. (1985) showed that the rate of lipolysis in vitro changed quadratically with respect to the amounts of starch and fiber in the incubation medium. They also found that rates of BH changed quadratically with respect to concentration of dietary starch and increased linearly with respect to dietary fiber. These findings may be related to the pH effect, or to the fact that triglycerides and FFA in the rumen are bound to the surface of feed particles and that lipolysis and BH occur at the lipid-feed particle binding site (Harfoot et al., 1973a,b, 1974, 1975). Thus, it would seem that accurate modeling of lipolysis and BH would necessarily have to take into account the nonlinear kinetic effects of pH and the kinetics of ruminal passage of feed particles.

It should also be recognized that most in vitro (batch and continuous culture) studies may not completely duplicate conditions normally occurring within the rumen. Hence, the nature of the kinetics observed and the magnitudes of the estimates for rates of lipolysis and BH obtained from these studies may be inaccurate. For example, the rumen fluid used in most in vitro studies is usually first strained through a filter and diluted with a buffer solution (Beam et al., 2000; Troegeler-Meynadier et al., 2003; Ribeiro et al., 2007). Thus, removal of feed particles (sites of lipolysis and BH) and dilution of enzyme concentrations could be expected to result in reduced estimates for rates of lipolysis and BH. Conversely, the feed substrates used in most in vitro experiments are usually first dried and ground (Beam et al., 2000; Ribeiro et al., 2007). Such processing could be expected to liberate intracellular lipids and therefore make them more susceptible to lipolysis and subsequent BH than lipids in feeds typically consumed by dairy cows. Therefore, discrepancies between rates of lipolysis and BH estimated from in vitro studies and rates calculated in vivo may, in large part, be attributed to the physical nature of the feed substrates investigated and how this affects the exposure of substrate lipids to rumen enzymes. These factors must therefore be taken into account if data and parameter estimates from in vitro experiments are to be used to model in vivo lipolysis and BH.

Ruminal lipolysis occurs because of the action of plant and bacterial lipases. It is believed that isomerization of linolenic acid to *cis*-9, *trans*-11, *cis*-15-18:3 must first occur before subsequent hydrogenation can occur. Similarly, linoleic acid must first be isomerized to *cis*-9, *trans*-11-CLA before subsequent BH can occur. Ruminal isomerization and hydrogenation occur because of the action of bacterial enzymes. Thus, because lipolysis, isomerization, and BH are all enzyme-mediated processes, it would seem logical that the kinetics of these processes should be described by traditional enzyme kinetics. Such enzyme kinetics almost always use a form of Michaelis-Menten kinetics. However, Michaelis-Menten kinetics has rarely been used to describe ruminal lipolysis or BH reactions.

To date, we are aware of 5 published attempts to model ruminal lipolysis and BH. Of these, the simplest model is a dynamic model incorporated into the Molly

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rumen submodel (Baldwin et al., 1987). However, this model has a fairly crude representation of lipid metabolism in the rumen and will not be considered further.

Dijkstra Model

A more advanced dynamic model of lipid metabolism in the rumen has been presented by Dijkstra et al. (2000; the Dijkstra model). In the Dijkstra model, there are 3 rumen lipid pools: unhydrolyzed lipid, saturated FFA, and unsaturated FFA. The fractional hydrolysis (lipolysis) rate of feed lipid is assumed to be 9 times the fractional solid passage rate. However, Dijkstra et al. (2000) suggested that if protected lipid is included in the diet, the fractional rate of lipolysis will have to be adjusted to 2.3 times the fractional solid passage rate. Dijkstra et al. (2000) also assumed that the maximal rate of BH was 9 times the fractional solid passage rate. In the Dijkstra model, BH is represented by a Michaelis-Menten-type equation in which the concentration of fiber in the rumen nonsigmoidally stimulates the BH rate, but the concentration of unsaturated fatty acids inhibits the BH rate in a sigmoidal way. Although the Dijkstra lipid model contains many interesting and novel features, the article by Dijkstra et al. (2000) described their lipid model in a qualitative fashion with an absence of equations and parameter values. Regrettably, since 2000 subsequent articles about this model do not appear to have been published, thus preventing its implementation and evaluation.

Ribeiro Model

Ribeiro et al. (2007) presented a dynamic model to describe the in vitro BH of unsaturated fatty acids derived from alfalfa (the Ribeiro model). In their model, the authors do not separate the kinetics of lipolysis from the kinetics of BH. They use first-order kinetics to describe the stepwise BH of fatty acids, beginning at *cis*-9, *cis*-12, *cis*-15-18:3 and culminating in the production of stearic acid. Their model appears to adequately describe the time course changes in a number of fatty acid intermediates. The alfalfa used in their investigation contained a relatively low concentration of fatty acids, so it is not known how well the model of Ribeiro et al. (2007) would predict these intermediates if the incubations were conducted with a substrate containing a high concentration of fat.

Moate Model

Moate et al. (2004) presented a static “production” model (the Moate model) of what is perhaps the first attempt to provide a relatively complete description of ruminal lipolysis and BH. In the Moate model, the rate of ruminal lipolysis of fat from each feed is described by separate rate constants, and estimates are provided for the lipolysis rates of fat in 25 common dairy feeds. The Moate model assumes that BH proceeds in the scheme 18:3 → 18:2 → *trans*-18:1 → 18:0. In addition, *cis*-18:1 is hydrogenated to 18:0 and 16:1 is hydrogenated to 16:0. This simplified approach does not account for the fact that the main pathways for BH involve specific positional isomers for each of the above-mentioned unsaturated fatty acids. This is because the in vivo data on which the parameters of the Moate model were estimated came from 8 experiments (involving 36 diets) that reported intakes and duodenal flows of all the major individual unsaturated fatty acids. Unfortunately, in these experiments, the unsaturated fatty acids were not identified by double bond position, and this necessitated the simplified, generic description of the fatty acids in the BH pathway.

In the Moate model, the fractional rates of BH of 18:3, 18:2, *trans*-18:1, and *cis*-18:1 are each described by separate exponential equations that depend on the concentration of unsaturated fatty acids present in the rumen. This exponential approach recognizes that rates of BH do not follow simple first-order kinetics. In addition, the model includes equations to predict the ruminal de novo production of fatty acids (16:0, 16:1, 18:0, and other fatty acids) from carbohydrate fermented in the rumen. The rates of passage out of the rumen of FFA are dependent on the solids passage rates of the feeds from which the fatty acids were derived. This is consistent with the fact that triglycerides and FFA in the rumen are mainly bound to the surface of feed particles (Harfoot et al., 1973b, 1974, 1975).

The Moate model was validated against an independent set of data from 8 experiments involving 36 diverse dietary treatments (Moate et al., 2004). Because the predictions of the Moate model were concordant with measured duodenal flows of all the main unsaturated fatty acids, the authors concluded that the Moate model described ruminal lipolysis and BH well. This rumen submodel provides the data that allow the subsequent estimation of the intestinal absorption of 10 individual fatty acids. A deficiency of this model is that it does not describe the influence of dietary fat intake on ruminal fiber digestion. Nevertheless, this model provides a conceptual framework for ruminal lipid modeling. Furthermore, because the Moate model is incorporated into CPM-Dairy (Moate et al., 2004), it can easily be used to conduct simulation experiments examining how inclusion of different fat supplements in diets affect the duodenal flows of the major fatty acids.

Harvatine and Allen Model

Recently, Harvatine and Allen (2006) used data from one experiment involving 4 dietary treatments to develop a static model of in vivo ruminal BH (Harvatine and Allen model). The main features of their model are that (1) there is no explicit rate constant to account for lipolysis; (2) it provides a simplified depiction of BH pathways in which 18:3 and 18:2 are hydrogenated directly to 18:1; and (3) rates of BH and lipolysis combined are assumed to be first order. Harvatine and Allen (2006) estimated rates of BH-lipolysis at between 28

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and 39%/h for linolenic acid, 15 and 17%/h for linoleic acid, 33 and 48%/h for *trans*-18:1, and 9 and 12%/h for oleic acid. Estimation of these rate constants depended on the coestimation of rates of ruminal passage for these fatty acids. Harvatine and Allen (2006) estimated that the rates of ruminal passage were between 6.9 and 7.1%/h for linolenic acid, 2.4 and 2.6%/h for linoleic acid, 5.0 and 7.1%/h for *trans*-18:1, 4.2 and 4.9%/h for oleic acid, and 7.6 and 9.6%/h for stearic acid. These quite different passage rates are surprising, given the fact that fatty acids are known to be bound to the surface of feed particles (Harfoot et al., 1974, 1975) and hence are expected to have the same passage rates as feed particles. Harvatine and Allen (2006) concluded that the main assumptions of their model still needed to be tested and that their model should be challenged with new experimental data.

In conclusion, considerable opportunities remain for researchers to develop novel, improved, and more sophisticated static and dynamic models of ruminal lipolysis and BH of unsaturated fatty acids. One barrier to this work appears to be that researchers often do not publish the types of data that facilitate the development of such models. A conclusion of this review is that for progress to be made in modeling the kinetics of ruminal lipolysis and BH, modelers should become involved at the planning stage of experiments rather than as an afterthought during data analysis.

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