Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria

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Abstract **Digesta samples from the ovine rumen and pure ruminal bacteria were incubated with linoleic acid (LA) in deuterium oxide-containing buffer to investigate the mechanisms of the formation of conjugated linoleic acids (CLAs). Rumenic acid (RA; *cis*-9,*trans*-11-18:2), *trans*-9,*trans*-11-18:2, and *trans*-10,*cis*-12-18:2 were the major CLA intermediates formed from LA in ruminal digesta, with traces of *trans*-9, *cis*-11-18:2, *cis*-9,*cis*-11-18:2, and *cis*-10,*cis*-12-18:2. Mass spectrometry indicated an increase in the n+1 isotopomers of RA and other 9,11-CLA isomers, as a result of labeling at C-13, whereas 10,12 isomers contained minimal enrichment. In pure culture, *Butyrivibrio fibrisolvens* and *Clostridium proteoclasticum* produced mostly RA with minor amounts of other 9,11 isomers, all labeled at C-13. Increasing the deuterium enrichment in water led to an isotope effect, whereby ^{1}H was incorporated in preference to ^{2}H. In contrast, the type strain and a ruminal isolate of *Propionibacterium acnes* produced *trans*-10,*cis*-12-18:2 and other 10,12 isomers that were minimally labeled. Incubations with ruminal digesta provided no support for ricinoleic acid (12-OH,*cis*-9-18:1) as an intermediate of RA synthesis. We conclude that geometric isomers of 10,12-CLA are synthesized by a mechanism that differs from the synthesis of 9,11 isomers, the latter possibly initiated by hydrogen abstraction on C-11 catalyzed by a radical intermediate enzyme.—**Wallace, R. J., N. McKain, K. J. Shingfield, and E. Devillard. **Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *J. Lipid Res.* 2007.** 48: **2247–2254.**

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Clinical, biomedical, and in vitro studies have provided evidence to suggest that isomers of conjugated linoleic acid (CLA) may confer potential benefits to long-term human health, including cancer prevention, reduced atherosclerosis risk, and improved immune response (1–3). Although early studies have examined the physiological effects of a mixture of CLA isomers containing *cis*-9,*trans*-11-CLA [rumenic acid (RA)] and *trans*-10,*cis*-12-CLA as major components, there is increasing evidence in animal models to suggest that the physiological effects of CLA are isomer-specific (2–4). Ruminant-derived foods are the major source of CLA in the diet, with RA as the major isomer (5, 6). Isomers of CLA are formed during the metabolism of linoleic acid (LA; *cis*-9,*cis*-12-18:2) in the rumen (7, 8). Measurements of ruminal outflow have indicated that RA derived from LA is typically the most abundant CLA, but a wide range of isomers are formed during ruminal metabolism of dietary polyunsaturated fatty acids (8, 9). Understanding the mechanisms underlying the synthesis of specific CLAs in the rumen is important in that ruminal outflow determines the supply of CLA available for incorporation into milk and tissues. Furthermore, several isomers of CLA formed during LA metabolism in the rumen are also known to induce physiological effects in the host ruminant. Postruminal infusion studies in lactating cows have shown that *trans*-10,*cis*-12-CLA inhibits milk fat synthesis (10) and that *trans*-9,*trans*-11-CLA (11) and *trans*-10,*trans*-12-CLA (12) decrease milk fat desaturase indices.

Cloning, crystallization, and structural analysis of the isomerase catalyzing the formation of *trans*-10,*cis*-12-CLA by *Propionibacterium acnes* has revealed the geometry of fatty acid binding to the enzyme and demonstrated a mode of action that involves hydride abstraction by enzyme-bound flavin adenine dinucleotide and the involvement of specific aromatic amino acid residues (13). Comparable information on the enzyme that forms RA is much more rudimentary. Several early studies using various substrates, inhibitors, and labeling enabled the metabolism of LA in the ruminal biohydrogenating bacterium *Butyrivibrio fibrisolvens* to be characterized (14–16). However, experiments were conducted with impure enzyme, because it proved impossible to purify the enzyme to homogeneity and retain activity. Based on studies with whole cells, it was recently proposed that RA formation from LA by lactic acid bacteria involves a hydration-dehydration mechanism via a 10-hydroxy,*cis*-12-18:1 intermediate (17). Such

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a mechanism has been eliminated as a possible route of RA synthesis from LA in *B. fibrisolvens* (16).

The present study investigated possible mechanisms responsible for CLA isomer formation in ruminal digesta and pure strains of several key ruminal bacteria by examining the incorporation of ^{2}H in conjugated intermediates during incubations with LA and ^{2}H-labeled water. The results of these incorporation experiments suggested the possibility that a hydration product of LA might be an intermediate in RA formation; hence, incubations were carried out with ricinoleic acid (12-OH,*cis*-9-18:1) and ruminal digesta and bacteria.

METHODS

Incubations with ruminal digesta

Animal experimentation was carried out under conditions governed by a license issued by the United Kingdom Home Office. Four adult, ruminally fistulated sheep received a diet composed (g/kg) of grass hay (300), rolled barley (415), soybean meal (175), molasses (100), and a proprietary mineral and vitamin supplement (10) fed as a total mixed ration. Daily rations (1 kg/day) were formulated to meet or exceed maintenance energy and protein requirements and were offered as two equal meals at 8:00 AM and 4:00 PM. Ruminal digesta samples (~200 g) were collected from each animal at 11:00 AM, strained through four layers of muslin cloth, and diluted in an equal volume of anaerobic 0.1 M potassium phosphate buffer, pH 7.0, prepared with 99.8% deuterium-enriched water (Norsk Hydro, Oslo, Norway). Five milliliters of diluted ruminal digesta were added to 12.5 × 1.6 cm glass tubes closed with screw caps fitted with butyl rubber septa (Bellco Biotechnology, Vineland, NJ). The tubes were maintained under CO_2 and incubated at 39°C. An emulsion of LA (Sigma) in water (25 mg/ml) was prepared by sonication, and 100 μl was added to each tube. A 1 ml subsample was removed, transferred into a clean tube, and heated at 100°C for 10 min. Further subsamples were removed at 1, 4, and 24 h and handled in the same manner. Subsamples were cooled and stored at −20°C for later fatty acid determinations.

The metabolism of ricinoleic acid [(*R*)-12-hydroxy-*cis*-9-18:1; Sigma] was determined using ruminal digesta from the same sheep. Twenty milliliters of strained digesta collected from all sheep was transferred to separate flasks (three per sheep), to which 200 μl of ethanol or 200 μl of ricinoleic acid (100 mg/ml) in ethanol was added. One milliliter subsamples were removed periodically into 1 ml of 0.5 M orthophosphoric acid, and the mixture was stored at −20°C. At the end of the experiment, a 1 ml aliquot was thawed and submitted for fatty acid analysis.

Bacteria and growth conditions

Four species of bacteria were used. *Butyrivibrio fibrisolvens* JW11 was originally isolated from sheep as a proteolytic species (18). *Clostridium proteoclasticum* P-18 is a recently identified stearic acid-producing bacterium isolated from grazing sheep (19). *Propionibacterium acnes* G449 was isolated in the same study and found to produce *trans*-10,*cis*-12-CLA from LA. These ruminal bacteria are held in the culture collection maintained at the Rowett Research Institute. A type strain of *P. acnes* (DSM 1897) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). All transfers and incubations were carried out under O_2-free CO_2 at 39°C in Hungate-type tubes (20). Inoculum volumes were 5% (v/v) of a fresh culture into 5 ml of medium. The media used in these experiments were based on the liquid form of M2 medium (21). One batch of medium was made up using unlabeled water, and the other was prepared with 50% (v/v) deuterium oxide. LA was added to a final concentration of 50 mg/l. Fatty acids were prepared as a separate solution, sonicated for 4 min in a small volume of medium, and added to the medium before dispensing and autoclaving. *B. fibrisolvens* and *C. proteoclasticum* were incubated at 39°C for 4 h and *P. acnes* was incubated for 96 h. Growth of bacteria was measured in triplicate from the increase in optical density at 650 nm of the control tubes using a Novaspec II spectrophotometer (Amersham Biosciences). One milliliter was removed for protein analysis and analysis of the enrichment in water. Thereafter, 100 μl of 19:0 (200 mg/l in methanol) was added, and tubes were stored at −70°C and subsequently freeze-dried.

An additional experiment was also carried out with *B. fibrisolvens* JW11 using the same procedures, with the exception that the medium was freeze-dried and reconstituted in 99.8% deuterium oxide.

The influence of ricinoleic acid on the growth of *B. fibrisolvens*, *C. proteoclasticum*, and *P. acnes* was determined by adding up to 50 μg/ml ricinoleic acid to M2 medium, inoculating 5% (v/v), and incubating at 39°C. Turbidity was determined as described previously. Fatty acid metabolism was determined by comparing fatty acids in the inoculated medium at 36 h with fatty acids in the uninoculated controls.

Fatty acid extraction and analysis

Extraction of total fatty acids was based on the method of Folch, Lees, and Sloane-Stanley (22), incorporating the modifications of Devillard et al. (23). Fatty acid methyl esters and 4,4-dimethyloxazoline (DMOX) derivatives were prepared and analyzed with a gas chromatograph-mass spectrometer consisting of an Agilent Technologies UK (Stockport, Cheshire, UK) gas chromatograph (6890) coupled to a quadrupole mass selective detector. The gas chromatograph was fitted with a 100 m fused silica capillary column (inner diameter, 0.25 mm) coated with a 0.2 μm film of cyanopropyl polysiloxane (Varian Analytical Instruments, Walton-on-Thames, Surrey, UK), and helium was the carrier gas (24). Enrichment in the *m/z* n+1 and n+2 isotopomers (molecular ion + 1 and + 2, respectively) was determined by GC-MS (24). Enrichment in water was determined by gas isotope ratio mass spectrometry. Sample water was diluted to a range suitable for measurement and then equilibrated with a standard hydrogen gas in the presence of 5% platinum-on-alumina (Aldrich Chemical Co., Ltd., Dorset, UK) (25). Measurements were made using a VG SIRA 10 (VG Isotech, Middlewich, UK) gas isotope ratio mass spectrometer fitted with a split flight tube and HD collector. Standard H^{3+} corrections were performed using dedicated software at the time of measurement. Data obtained from the instrument were normalized against two international standards, Vienna-Standard Mean Ocean Water and Standard Light Arctic Water, according to standard procedures (26). The position of deuterium labeling was determined based on GC-MS analysis of DMOX derivatives.

Data analysis

Replicate measurements (n = 3) from incubations with rumen digesta from four sheep were averaged and analyzed by ANOVA for repeated measures using a model that included the fixed effect of incubation time and random effects of the animal assuming a compound symmetry covariance structure. Least-square means ± SEM are reported, and effects were considered significant at $P < 0.05$. Enrichment of n+1 isotopomers was calculated from the *m/z* ratios at n, n+1, n+2, and n+3 by deconvolution

according to Campbell (27). Natural abundance was calculated from the isotopomer distribution of LA in zero time samples. The significance of differences between fatty acid concentrations and enrichments in the high-enrichment versus zero-enrichment cultures of *B. fibrisolvens* with LA was evaluated by an unpaired *t*-test (n = 3).

RESULTS

Formation of CLA in ruminal digesta diluted in deuterium oxide

Strained ruminal digesta was collected from four mature sheep and diluted with an equal volume of buffer prepared using deuterium oxide. LA was added and the mixtures were incubated anaerobically in vitro for up to 24 h (**Fig. 1**). LA concentration declined rapidly, resulting in the accumulation of six CLA isomers. RA was the most abundant isomer, with lower concentrations of *trans*-10,*cis*-12-CLA and *trans*-9,*trans*-11-CLA also being formed (Fig. 1A). Minor amounts of *trans*-9,*cis*-11-CLA, *cis*-9,*cis*-11-CLA, and *cis*-10,*cis*-12-CLA were also detected (Fig. 1B). Concentrations of RA were highest after 4 h, declining thereafter by ~50% between 4 and 24 h. In contrast, the concentration of other CLA isomers increased over the same period.

Mass spectra of methyl esters in samples collected after 24 h incubations indicated that 9,11 geometric isomers of CLA were labeled at n+1. Independent analysis of the enrichment in water allowed the ratio of MPE (moles percent excess) in fatty acid ester peaks to be compared with the MPE of water. All samples of water had a similar enrichment (43.4 ± 0.09%; mean ± SEM). Calculated labeling (MPE sample/MPE water) averaged 0.618 for 9,11 geometric CLA isomers (**Fig. 2**). Labeling at n+2 was 0.069. In contrast, labeling at n+1 and n+2 for *trans*-10,*cis*-12-CLA was 0.118 and 0.013, respectively. There was no evidence of differences in labeling at n+1 in samples collected at 1 and 24 h (Fig. 2). Concentrations of other CLA isomers at 1 h were insufficient to allow the MPE at n+2 to be estimated accurately, but the MPE ratio of n+2 was 0.007 ± 0.004 and 0.013 ± 0.002 for RA and *trans*-10,*cis*-12-CLA, respectively.

The mass spectrum of the DMOX derivative of *cis*-9,*trans*-11-CLA (**Fig. 3**) indicated enrichment in ion fragments from the molecular ion to *m/z* 262. The occurrence of ion fragment isotopomers with $m/z < 262$ was comparable to the natural abundance of ~20% MPE. This analysis provides unequivocal evidence that deuterium was labeled on C-13 of the fatty acid moiety (Fig. 3). Mass spectra of other 9,11 geometric CLA isomers revealed a similar pattern of enrichment and that the small amount of labeling for *trans*-10,*cis*-12-18:2 was also located on C-13.

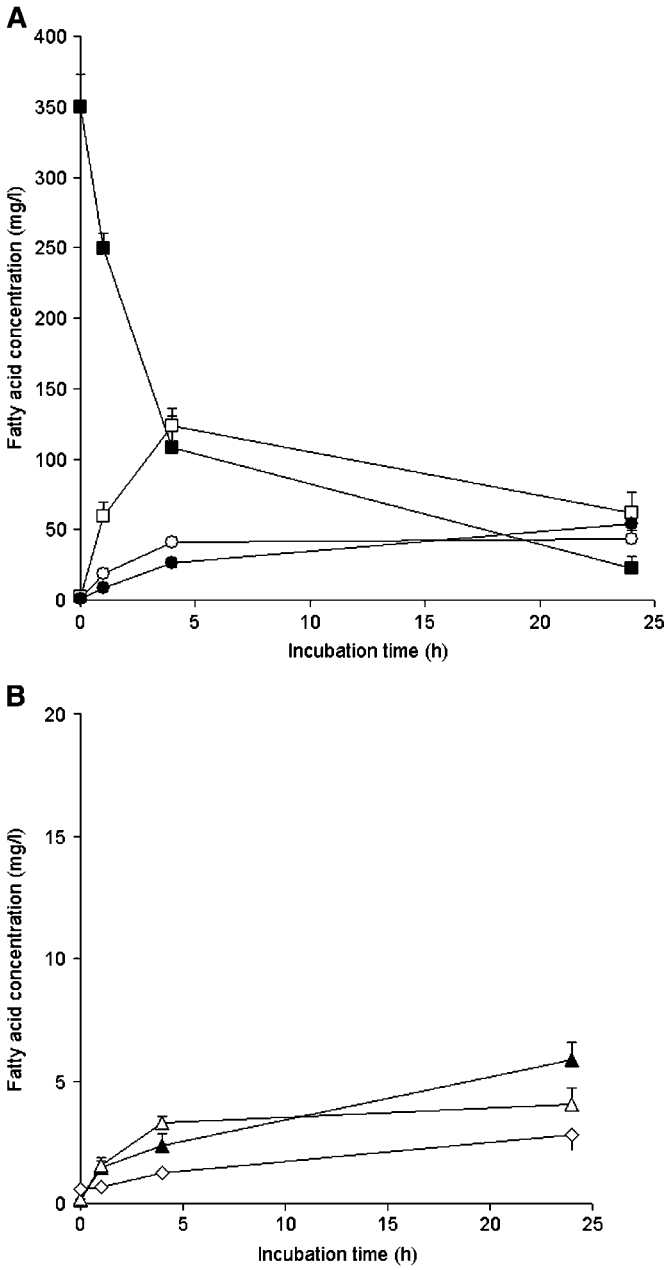

Fig. 1. Synthesis of conjugated linoleic acid (CLA) isomers from linoleic acid (LA) during 24 h incubations with mixed ovine ruminal microorganisms. Strained ruminal digesta was collected from the rumen of four sheep, diluted in an equal volume of buffer prepared with 43.4 ± 0.09% MPE (moles percent excess) deuterium oxide, and incubated at 39°C in the presence of 500 mg LA/l. A: LA (closed squares), *cis*-9,*trans*-11-CLA (open squares), *trans*-10,*cis*-12-CLA (open circles), *trans*-9,*trans*-11-CLA (closed circles). B: *trans*-9,*cis*-11-CLA (closed triangles), *cis*-9,*cis*-11-CLA (open triangles), and *cis*-10,*cis*-12-CLA (open diamonds). Mean incubation protein content was 3.65 ± 0.26 mg/ml. Values are means ± SEM (n = 4).

Formation of CLA by pure cultures of ruminal bacteria

Four CLA-producing bacteria were grown in the presence of 50 mg/l LA in deuterium oxide-enriched medium, and samples were collected for fatty acid determinations during the exponential phase of growth for *B. fibrisolvens* and *C. proteoclasticum* and in the stationary phase for both *P. acnes* strains. Samples from incubations with *B. fibrisolvens* and *C. proteoclasticum* were taken at the exponential phase to avoid further reduction of CLA isomers to 18:1 metabolites or 18:0 end product in the case of *C. proteoclasticum*. Results for *B. fibrisolvens* and *C. proteoclasticum* were similar, with RA being the main CLA isomer

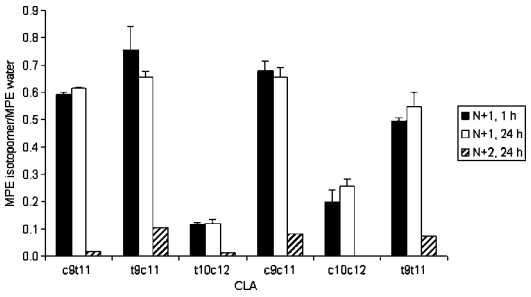

Fig. 2. Enrichment in n+1 and n+2 isotopomers of CLA isomers synthesized from LA during incubations with mixed ovine ruminal microorganisms. Mean enrichment in water was 43.4 ± 0.09% MPE. Values are means ± SEM (n = 4).

followed by *trans*-9,*trans*-11-CLA (**Table 1**). No 10,12 isomers of CLA were detected in the incubations of LA with either species.

The MPE enrichment of deuterium in the medium water was determined as 42.8 ± 0.90%. All 9,11 CLA isomers produced by *B. fibrisolvens* and *C. proteoclasticum* were labeled, whereas 10,12 geometric CLA isomers formed by both the type strain of *P. acnes* (DSM 1897) and the ruminal isolate (G449) were minimally labeled (Table 1). Calculated MPE ratios relative to enrichment in water varied between 0.54 and 0.59 (Table 1), with the positioning of labeling determined to be at C-13 of the fatty acid moiety.

B. fibrisolvens was also grown in medium containing a much higher enrichment of deuterium oxide and in medium with water of natural abundance to exclude possible isotope effects on fatty acid metabolism. Even though reconstituted dried medium with water was enriched by 99.8% MPE with deuterium, the enrichment measured in the final medium was 96.1% MPE. Metabolism of LA occurred at a slower rate in the deuterium oxide medium, as shown by the lower concentration of RA at 4 h (**Table 2**). Enrichment in CLA isomers was found to be lower than that in water, averaging 0.81 for all four 9,11 geometric CLA isomers.

Ricinoleic acid metabolism by ruminal microorganisms

The metabolism of ricinoleic acid was investigated using mixed digesta collected from four sheep (**Fig. 4**). Ricinoleic acid was metabolized at a lower rate than LA. No CLA or 18:1 intermediates were formed. Concentrations of several late-eluting peaks were found to be increased after incubations with ricinoleic acid, but only one, 10-hydroxy-18:0, could be identified unequivocally.

Ricinoleic acid proved to be highly toxic to ruminal bacteria, and none of the four bacteria used here was capable of growing in medium containing 50 mg/l ricinoleic acid.

DISCUSSION

Biomedical studies with animal models have shown that CLA exerts a wide range of physiological responses, and there is increasing evidence that the effects are isomer-specific (8, 28–31). The development of biotechnology systems for the synthesis of single isomer preparations would offer significant advantages over chemical methods that result in the production of materials containing a range of CLA isomers. Therefore, understanding the mechanisms underlying the synthesis of specific CLA isomers in biological systems is important. The present research provides strong evidence that the synthesis of RA from LA by ruminal bacteria occurs via a different biochemical mechanism than *i*) CLA synthesis from LA by *Propionibacterium* and *Lactobacillus* species and *ii*) the formation of *trans*-10,*cis*-12-CLA from LA in the rumen.

Several bacterial species are known to synthesize RA from LA. The first ruminal bacterium reported to produce RA was *B. fibrisolvens* (32). Further studies (33) have indicated that the high capacity to metabolize LA to RA is associated entirely with the *Butyrivibrio* group, which includes stearate-producing *C. proteoclasticum*, previously named *Fusocillus* (34, 35). Among nonruminal bacteria, *Propionibacterium freudenreichii* subspecies *freudenreichii* forms mainly 9,11 geometric isomers of CLA from LA (23, 36). Even though *Lactobacillus* and *Lactococcus* species were reported to be incapable of CLA synthesis in some studies (23, 36, 37), other experiments demonstrated that the ability to produce CLA from LA, including RA, is widely distributed among lactic acid bacteria (17, 38), including *Lactobacillus* and *Propionibacterium* species. The synthesis of RA from LA is also associated with *Bifidobacterium* species (23, 37) and some human intestinal *Roseburia* (23). Overall, the specific activity for RA production from LA is severalfold higher in *Butyrivibrio*-related ruminal bacteria compared with nonruminal species (7, 33, 39).

Fewer bacterial species are known to synthesize *trans*-10,*cis*-12-CLA from LA. The isomerase responsible for *trans*-10,*cis*-12-CLA formation by a human skin-derived *P. acnes* has been characterized extensively (13). *Trans*-10,*cis*-12-CLA-producing *P. acnes* isolates have also been isolated from the rumen (33). A strain of *Megasphaera elsdenii* isolated from the rumen has been reported to produce *trans*-10,*cis*-12-18:2 (40), although in our laboratory no strain of *M. elsdenii*, including the type strain, was found to produce CLA from LA; therefore, *P. acnes* was used in the present study. Among human intestinal isolates, bifidobacteria produce trace amounts of *trans*-10,*cis*-12-CLA (23, 37). *Lactobacillus rhamnosus* was reported to produce *trans*-10,*cis*-12-CLA (41), whereas *Lactobacillus acidophilus* and *Lactobacillus casei* produce *trans*-10,*cis*-12-CLA as a minor product during LA metabolism (38). No other *Lactobacillus* or *Lactococcus* species have been identified as capable of *trans*-10,*cis*-12-CLA synthesis (17, 42–44).

Milk fat contains a diverse range of CLA isomers (8, 45–47). Other than isomers containing a *cis*-9 double bond, it is reasonable to assume that these originate from the rumen, but the microorganisms and enzymes responsible are not well characterized. Undoubtedly, extensive interspecies exchange of fatty acids must occur in the rumen, leading to a wide range of biohydrogenation metabolites, but the present results confirm that pure cultures synthesize several CLA isomers during LA metabolism. In

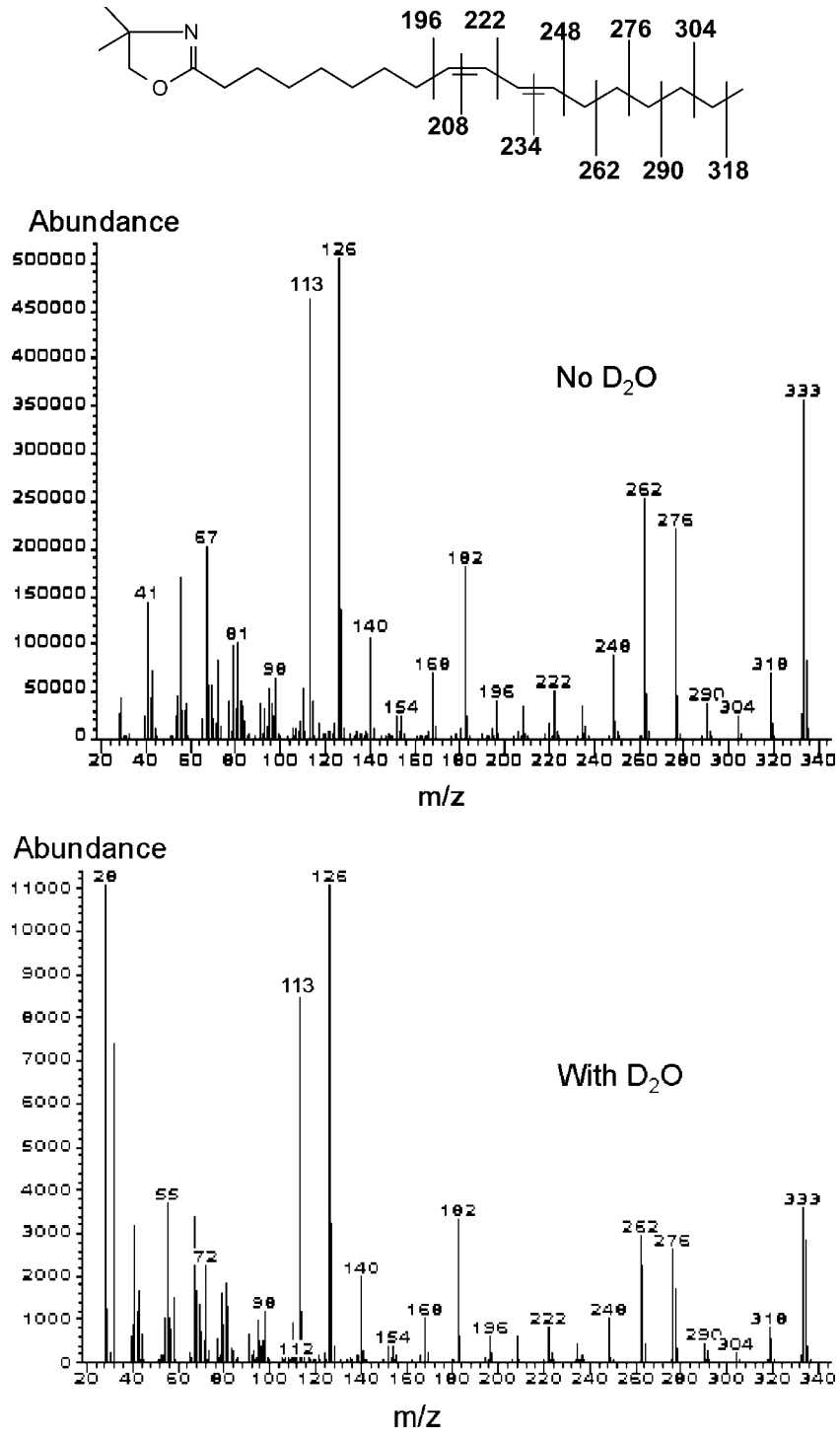

Fig. 3. Mass spectrum of the 4,4-dimethyloxazoline derivative of *cis*-9,*trans*-11-18:2 synthesized from LA during 4 h incubations with mixed ovine ruminal digesta. Enrichment of ion fragments at *m/z* 262 and 263 indicate that ^{2}H is located on C-13 of the fatty acid moiety.

this study, *B. fibrisolvens* was shown to form a conjugated diene in addition to RA, confirming earlier observations (32), which was identified unequivocally as *trans*-9,*trans*-11-CLA. *Trans*-9,*trans*-11-CLA is the major CLA synthesized from LA by some lactobacilli, but the products are highly variable between species (17, 23, 37, 41–43). *P. acnes* also produces 10,12 geometric isomers of CLA other than *trans*-10,*cis*-12-CLA. The balance of isomers produced in the mixed ruminal community is thought to be altered by pH (48), possibly because different species have different pH ranges for optimal growth.

The kinetics of CLA production differed for the different isomers in ruminal digesta, with RA and *trans*-10,*cis*-12-CLA concentrations decreasing between 4 and 24 h, whereas the

TABLE 1. Synthesis of CLAs from LA by pure cultures of bacteria inoculated into medium enriched with deuterium oxide-enriched water and enrichment in n+1 isotopomers

	cis-9,*trans*-11		*trans*-9,*cis*-11		*trans*-10,*cis*-12		*cis*-9,*cis*-11		*cis*-10,*trans*-12		*trans*-9,*trans*-11		*trans*-10,*trans*-12	
Bacterium and Measure	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Concentration (mg/l)														
B. fibrisolvens	32.1	1.90	0.8	0.11	0	0.00	1.5	0.10	0	0.00	8.3	0.55	0	0.00
C. proteoclasticum	29.7	1.01	0.8	0.03	0	0.00	1.5	0.09	0	0.00	8.4	0.41	0	0.00
P. acnes DSM 1897	0.4	0.01	0	0.00	25.2	0.45	0	0.00	1	0.01	0	0.00	5.6	0.10
P. acnes G449	0.3	0.01	0	0.00	21.7	0.18	0	0.00	0.8	0.03	0	0.00	3.7	0.61
MPE ratio														
B. fibrisolvens	0.577	0.0092	0.539	0.0150	0	0.0000	0.570	0.0150	0	0.0000	0.579	0.0064	0	0.0000
C. proteoclasticum	0.584	0.0104	0.574	0.0422	0	0.0000	0.590	0.0191	0	0.0000	0.591	0.0017	0	0.0000
P. acnes DSM 1897	0	0.0000	−0.054	0.0029	0.023	0.0012	0	0.0000	0.048	0.0040	0	0.0000	0.077	0.0058
P. acnes G449	0	0.0000	−0.083	0.0607	0.033	0.0058	0	0.0000	0.021	0.0029	0	0.0000	0.036	0.0179

CLA, conjugated linoleic acid; LA, linoleic acid; MPE, moles percent excess. The initial concentration of LA was 50 mg/l. For deuterium oxide-enriched water, mean was 42.8% MPE enrichment in water. *B. fibrisolvens* and *C. proteoclasticum* were 4 h exponential phase cultures and *P. acnes* were 24 h stationary phase cultures. The MPE ratio indicates MPE in CLA isomer/MPE in water. n = 3.

TABLE 2. Synthesis of CLA isomers in 4 h cultures of *B. fibrisolvens* growing in medium containing 96.1% deuterium-enriched water and 50 mg/l LA

	Concentration				MPE Ratio	
	Water		Deuterium Oxide		Deuterium Oxide	
Isomer	Mean	SEM	Mean	SEM	Mean	SEM
	mg/l					
Cis-9,*cis*-12-18:2	1.5	0.7	6.2[a]	1.8	0.028	0.006
Cis-9,*trans*-11-CLA	32.1	0.8	23.6[b]	3.3	0.839	0.007
Trans-9,*cis*-11-CLA	0.7	0.2	0.6	0.1	0.785	0.066
Cis-9,*cis*-11-CLA	0.9	0.2	0.8	0.1	0.800	0.022
Trans-9,*trans*-11-CLA	4.1	0.9	3.3	0.7	0.823	0.013

The MPE ratio indicates MPE in CLA isomer/MPE in water. n = 3.
[a] Different at $P < 0.001$.
[b] Different at $P < 0.005$.

concentrations of the minor CLA isomers continued to increase. RA is converted rapidly to *trans*-11-18:1, which explains the decline in RA concentration over time. *Trans*-10,*cis*-12-CLA is thought to be reduced to *trans*-10-18:1, whereas the fate of the other CLAs is not known. It is possible that they are metabolized to 18:1 products more slowly than RA.

The enrichment in *cis*-10,*cis*-12-CLA was apparently higher than that in *trans*-10,*cis*-12-CLA (Fig. 2), but the concentration of the former was extremely low, so the enrichment was difficult to estimate. Owing to the low concentrations, it remains possible that the *trans*-9,*trans*-11-CLA peak also contained *trans*-10,*trans*-12-CLA as a minor component, which would serve to reduce the enrichment of this intermediate.

The level of understanding of the mechanisms of enzyme-catalyzed reactions leading to the formation of

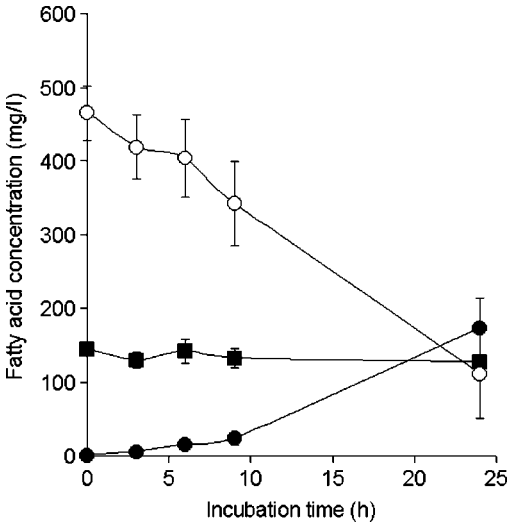

Fig. 4. Metabolism of ricinoleic acid by mixed ruminal microorganisms. Strained ruminal digesta from the rumen of four sheep was incubated at 39°C in the presence of 1 g/l ricinoleic acid. Ricinoleic acid (open circles), 10-hydroxy-18:0 (closed circles), and stearic acid (closed squares). Results are means ± SEM from four sheep. The protein content of the incubation mixtures was 6.81 ± 1.32 mg/ml. Concentrations of LA, CLA isomers, *trans*-10-18:1, and *trans*-11-18:1 were <10 mg/l in all samples.

CLA isomers differs across species and specific isomers. The determination of crystalline structures has provided detailed information on the formation of *trans*-10,*cis*-12-CLA from LA for *P. acnes* (13). Based on stereochemical considerations, it has been postulated that isomerization occurs via an ionic reaction, whereby isomerization is initiated via hydride transfer from C-11 to the N5 of bound flavin adenine dinucleotide, followed by electron migration resulting in the formation of a carbocation and reintroduction of a hydride on C-9 of the fatty acid. Such a mechanism does not involve an exchange with water, consistent with the low incorporation of ^{2}H from deuterium oxide in the present experiments with *P. acnes*. This study also provides strong evidence that the same mechanism described for *P. acnes* is also responsible for *trans*-10,*cis*-12-CLA synthesis from LA by the mixed microbial community in the rumen. Furthermore, other 10,12 geometric isomers formed by *P. acnes* during incubation with LA were also unlabeled. However, it is not possible to deduce whether the less abundant 10,12 isomers are formed as products of the same enzyme-catalyzed reaction or synthesized from subsequent *cis-trans* isomerization of *trans*-10,*cis*-12-CLA.

The mechanism responsible for RA formation is much less clear, partly because of the difficulty of purifying enzymes from *B. fibrisolvens* (16) and *Lactobacillus* species (49). A complicated mechanism based on a hydration-dehydration reaction and two successive *cis-trans* isomerizations was proposed for RA synthesis by *L. acidophilus* based on the accumulation of 10-hydroxy-*cis*-12-18:1 during incubations with LA (17, 44). Early studies were unable to detect the formation of hydroxy intermediates during the isomerization of LA in *B. fibrisolvens* (16). Even though the incorporation of a hydrogen atom from water at C-13 of RA, as determined in this and earlier studies (14), may implicate ricinoleic acid as a putative intermediate of a hydration-dehydration reaction via the initial hydration of the Δ^{12} bond, ricinoleic acid was not converted to CLA by *B. fibrisolvens* (16). The present data also revealed that the incorporation of a hydrogen atom from water at C-13 of RA also occurred during incubations of LA with mixed ruminal digesta, but crucially, ricinoleic acid was reduced to hydroxy fatty acid intermediates and not to isomers of CLA. In contrast, lactobacilli are known to synthesize RA from ricinoleic acid (17, 42). Measurements of ^{2}H enrichment indicate that the mechanism of RA synthesis by ruminal bacteria differs from that responsible for RA formation in *Lactobacillus* and *trans*-10,*cis*-12-18:2 synthesis by *P. acnes*.

Previous studies noted that 11,11-dideuterolinoleic acid was isomerized at 50% of the rate of unlabeled LA (16). The K_m was unaffected, but the V_{max} decreased. Coupled with additional observations, Kepler, Tucker, and Tove (16) proposed that the isomerization of LA proceeded via proton removal from C-11 and a stereospecific attack by the proton of a conjugate acid in equilibrium with water. Based on current data, we suggest that the activity of linoleate isomerase of *B. fibrisolvens* acting as a radical intermediate enzyme is a more likely explanation. It should be noted that observations of radical intermediate enzyme activity were rare at the time when the original studies (16) were conducted. We now know that several families of radical enzymes occur in anaerobes and to a lesser extent aerobic organisms, including coenzyme B_{12}-dependent eliminases and mutases and *S*-adenosylmethionine radical enzymes, such as lysine-2,3-aminomutase from *Clostridium* species (50).

Therefore, it is plausible that the H on C-11 of LA is removed by hydrogen abstraction, leaving a radical that is thermodynamically less favorable than a conjugated double bond system with the radical located on C-13. Thus, the movement of the double bond from carbon atoms 12 and 13 proceeds by reasons of thermodynamic stability. To complete the reaction, a hydrogen atom is then abstracted from water. The proposed mechanism may explain why the reaction kinetics of RA formation are so unusual (49, 51) and why a cofactor is required (E. Devillard, unpublished data). It is also possible that the formation of other 9,11 conjugated isomers is marginally less energetically favorable than RA for the stabilization of the radical intermediate. Such a mechanism also implies that the formation of 9,11 geometric isomers occurs simultaneously and does not involve subsequent *cis-trans* or *trans-cis* isomerization of RA. Further measurements based on NMR would be required to confirm unequivocally that this mechanism is responsible for the synthesis of 9,11-CLA isomers from LA by ruminal bacteria.

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