Isomerization of stable isotopically labeled elaidic acid to *cis* and *trans* monoenes by ruminal microbes

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Abstract A previous study showed that oleic acid was converted by mixed ruminal microbes to stearic acid and also converted to a multitude of *trans* octadecenoic acid isomers. This study traced the metabolism of one of these *trans* C18:1 isomers upon its incubation with mixed ruminal microbes. Unlabeled and labeled (18-[^{13}C]*trans*-9 C18:1) elaidic acid were each added to four in vitro batch cultures with three cultures inoculated with mixed ruminal bacteria and one uninoculated culture. Samples were taken at 0, 12, 24, and 48 h and analyzed for ^{13}C enrichment in component fatty acids by gas chromatography-mass spectrometry. At 0 h of incubation, enrichment was detected only in elaidic acid. By 48 h of incubation, ^{13}C enrichment was 18% ($P < 0.01$) for stearic acid, 7% to 30% ($P < 0.01$) for all *trans* C18:1 isomers having double bonds between carbons six through 16, and 5% to 10% for *cis*-9 and *cis*-11 monoenes. After 48 h, ^{13}C enrichment in the uninoculated cultures was only detected in the added elaidic acid. This study shows *trans* fatty acids exposed to active ruminal cultures are converted to stearic acid but also undergo enzymic isomerization yielding a multitude of positional and geometric isomers.— Proell, J. M., E. E. Mosley, G. L. Powell, and T. C. Jenkins. **Isomerization of stable isotopically labeled elaidic acid to *cis* and *trans* monoenes by ruminal microbes. *J. Lipid Res.* 2002.** 43: **2072–2076.**

Supplementary key words isomerization • elaidic acid • *trans* monoenes • ruminal microbes

Anaerobic bacteria that colonize the rumen, or largest of the four stomach compartments in ruminant species, carry on a process of lipid biohydrogenation whereby double bonds in unsaturated fatty acids are partially or completely eliminated. Linoleic acid disappeared completely by 50 h when incubated with mixed ruminal microorganisms (1). As linoleic acid disappeared, transient increases in a number of *trans* diene isomers were seen, followed by the accumulation of *trans*-11 C18:1. During the later hours of incubation, the *trans*-11 C18:1 declined slowly and was accompanied by an increase in stearic acid concentration (1).

Oleic acid biohydrogenation is generally presented as a direct conversion to stearic acid without the formation of *trans* intermediates (1, 2). When ^{13}C-labeled oleic acid was incubated with ruminal microorganisms in a recent study (3), enrichment was observed not only in stearic acid but also in all *trans* C18:1 isomers having double bonds at carbon positions six through 16. However, the fate of these positional isomers of *trans*-C18:1 is not clear. *Trans*-11 C18:1 is readily converted to stearic acid by select ruminal bacteria (4), but its conversion to other *trans* monenes has not been reported.

Kemp et al. (5) incubated *cis* (*cis*-2 and *cis*-4 to *cis*-13) and *trans* (*trans*-2 and *trans*-5 to *trans*-13) octadecenoic acid isomers with a rumen *Fusocillus* species. They wanted to test the ability of *Fusocillus* to hydrogenate the octadecenoic acids 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.

This study was conducted to determine the fate of carbons from *trans*-9 C18:1 (elaidic acid) following its incubation with mixed ruminal microbes for 48 h. Cultures of mixed ruminal microbes were supplemented with ^{13}C-labeled elaidic acid to determine possible enrichment in stearic acid and other monene isomers.

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Abbreviations: APE, atom percent excess; DMDS, dimethyl disulfide; FAME, fatty acid methyl ester; GC-MS, gas chromatography-mass spectrometry.

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MATERIALS AND METHODS

Reagents

Labelled elaidic acid (18-[^{13}C]*trans*-9 C18:1) was purchased from CDN Isotopes (Quebec, Canada). Unlabelled elaidic acid (99% pure) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All solvents were HPLC or GC grade. Dimethyl disulfide (DMDS), silver nitrate crystal, anhydrous ethyl ether, iodine, and sodium thiosulfate were purchased from Fisher Scientific (Pittsburgh, PA).

Microbial cultures

Microbial conversion of elaidic acid was studied in cultures of mixed gut microbes taken from the stomach (rumen compartment) of cattle. Cultures were maintained in 125 ml Erlenmeyer flasks containing 500 mg of ground hay, 40 ml of media, 50 mg of elaidic acid, and 2 ml of reducing solution according to Goering and Van Soest (6). Unlabelled cultures received 400 μl of elaidic acid in ethanol (125 mg/ml). The labeled cultures received 400 μl of an elaidic acid solution in ethanol (125 mg/ml) consisting of 50% 18-[^{13}C]elaidic acid and 50% unlabelled elaidic acid. Cultures containing unlabeled or labeled elaidic acid ($n = 4$) were run at 39°C under anaerobic conditions.

Three of the four labeled and unlabeled flasks were inoculated with microbes collected from the rumen of a fistulated Holstein cow. Contents from the rumen were thoroughly mixed by hand 2 h after the morning feeding, strained through two layers of cheesecloth, and added (10 ml) to culture flasks while gassing continuously with CO_2. The remaining labeled and unlabelled flask received an additional 10 ml of media in place of the ruminal inocula.

Duplicate samples (5 ml) were taken from each culture at 0 h, 12 h, 24 h, and 48 h and immediately frozen. The samples were freeze-dried and then methylated according to Kramer et al. (7). When stored, all samples containing fatty acid methyl esters (FAME) were stored in an organic solvent at −15°C.

Solid phase extraction column separation

The FAME samples from each incubation time were taken to dryness under a stream of nitrogen gas and then dissolved in 0.4 ml of methylene chloride. The FAME were separated into saturated, *trans* monoene, *cis* monoene, and diene fractions using a modified procedure of Christie (8). The modified procedure is as follows: an Isolute® SCX-2 (International Sorbent Technology, Mid Glamorgan, UK) solid phase extraction column (500 mg, 10 ml reservoir) was wrapped to the level of the top of the absorbent bed with aluminum foil. The column was preconditioned by elution with 2 ml of acetonitrile. A solution of 20 mg of silver nitrate in 0.25 ml acetonitrile-water 10:1 (v/v) was allowed to flow through the solid phase extraction column. The column was flushed with acetonitrile (5 ml), acetone (5 ml), and methylene chloride (10 ml). The FAME sample in 0.4 ml methylene chloride was divided equally between two columns for better resolution and washed onto the column in methylene chloride (0.2 ml). Saturated fatty acids were eluted with methylene chloride (5 ml). The monoene fraction was separated into *trans* monoenes and *cis* monoenes by washing with 0.5% acetone in methylene chloride (5 ml) and 10% acetone in methylene chloride (5 ml), respectively. Dienes were eluted with acetone (5 ml). All fractions were eluted by gravity. Corresponding fractions from the two columns were combined and taken to dryness under a stream of nitrogen gas. The FAME in the saturated and diene fractions were dissolved in 200 μl of hexane and analyzed by gas chromatography-mass spectrometry (GC-MS).

DMDS derivatization

DMDS adducts of the *trans* monoene and *cis* monoene fractions were prepared using a modified procedure of Yamamoto et al. (9). The modified procedure is as follows: the FAME fractions were treated with 0.35 ml of DMDS and 100 μl of iodine solution (6% iodine w/v in diethyl ether). The reaction mixtures were shaken in a 37° C water bath for 1 h and then diluted with diethyl ether-hexane (3 ml; 1:1, v/v). Iodine was removed by shaking with 10% sodium thiosulfate (200 μl). The organic phase was removed and the solvent was evaporated under a stream of nitrogen gas. The residue was dissolved in 200 μl of hexane and analyzed immediately by GC-MS. When stored, samples were stored no longer than 2 days in hexane at −15°C.

GC-MS

Analysis of the FAME in the saturated and diene fractions, and the DMDS derivatives in the *trans* and *cis* fractions were analyzed by GC-MS as described by Mosley et al. (3). Additional FAME samples containing 1 mg of C17:0 internal standard were analyzed on a gas chromatograph (Shimadzu GC-14A; Columbia, Maryland) equipped with a flame ionization detector and a 100 m × 0.25 mm, with 0.2 μm film capillary column coated with CP-Sil 88 (Chrompack, Raritan, New Jersey). The injector and detector temperatures were both 250°C. The carrier gas was H_2 (33 cm/s) with an inlet pressure of 250 kPa. The column temperature was isothermal at 160°C (held for 45 min) to separate major fatty acids.

Calculations and statistics

The DMDS derivatives of FAME produce two distinctive spectral fragments that are indicative of the double bond position when analyzed by mass spectrometry. The F fragment is the methyl thio adduct of the methyl end of the FAME. The G fragment is the methyl thio adduct of the carboxyl end of the FAME. The atom percent excess (APE) was calculated from the mass abundance of the F and F + 1 fragments using the equation $APE = (F + 1)/[F + (F + 1)]$. In order to correct for the natural levels of ^{13}C, the average APE of unlabeled cultures was subtracted from the APE of labeled cultures. Therefore, enrichment of the fatty acid with ^{13}C was calculated as $(APE_{\text{labeled}} - \text{average } APE_{\text{unlabeled}})*100$.

Changes in fatty acid concentration (mg/5 ml culture) over time were determined by analysis of variance using the PROC GLM (general linear model) procedure of SAS (SAS Institute, Inc., Cary, NC). Means and standard deviations were determined by the PROC MEAN procedure of SAS, with enrichment analyzed by Student's *t*-test to determine if they differed from zero.

RESULTS AND DISCUSSION

Total fatty acid concentration in the cultures increased ($P < 0.05$) from 0 h to 12 h of incubation (4.80 to 5.23 mg/5 ml) then remained constant through 48 h (**Fig. 1**). The slight increase in total fatty acids over time is due to the lack of fatty acid catabolism by ruminal anaerobes combined with their ability to synthesize long-chain fatty acids de novo from fermentation acids (4). The concentration of elaidic acid declined ($P < 0.05$) over time. Unsaturated fatty acids exposed to ruminal microbes generally decrease in concentration due to their biohydrogenation to more saturated end products. Stearic acid concentration increased ($P < 0.05$) over time, but the concentrations of C16:0 and *cis*-9 C18:1 were small at 0 h and changed little.

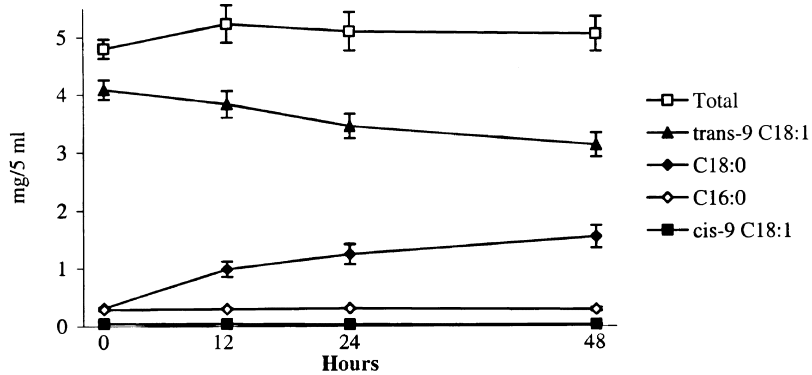

Fig. 1. Changes in total fatty acid concentration (mg/5 ml of culture) and concentrations of major fatty acids over time in cultures of ruminal bacteria supplemented with elaidic acid. Points are the means of four replicates with standard deviations.

The enrichment of C18:0 at 12 h through 48 h (**Fig. 2**) supports biohydrogenation as the process for the conversion of elaidic acid to C18:0, and accounts for the disappearance of *trans*-9 C18:1 over time. Earlier work (5) also showed the conversion of elaidic acid to C18:0 by a ruminal *Fusocillus* species. Mosley et al. (3) recently confirmed that carbons from oleic acid were transferred to stearic acid plus a number of positional isomers of *trans* monoenes in cultures of mixed ruminal microbes. This study extended those observations by showing that one of those positional isomers, namely *trans*-9 C18:1, was converted to stearic acid. When the results of the two investigations are

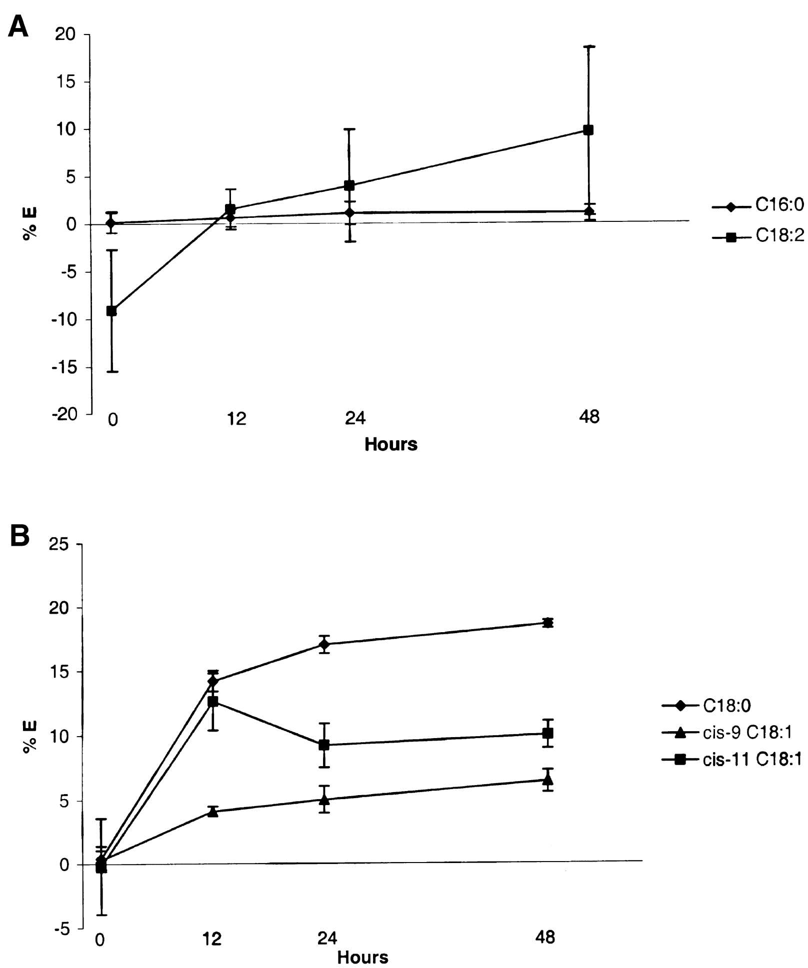

Fig. 2. The percentages of ^{13}C enrichment in (A) C16:0 and C18:2 and (B) C18:0, *cis*-9 C18:1, and *cis*-11 C18:1 over time when a mixture (1:1) of elaidic and 18-^{13}C-elaidic acids were added (50 mg per culture) to cultures of mixed ruminal microbes. Each point is the mean of three replicates with standard deviations.

taken together, they suggest a biohydrogenation pathway for oleic acid that is more similar to linoleic acid than is usually stated. Linoleic acid is acted upon by an isomerase yielding several *trans* conjugated dienes, which in turn are reduced to *trans* monoene intermediates with *trans*-11 C18:1 being the most abundant (4). Conversely, the biohydrogenation of oleic acid, as it is usually depicted, proceeds directly to stearic acid without the action of an isomerase or the accumulation of *trans* monenes. The results of the current study and the previous study by Mosley et al. (3) indicate the presence of one or more isomerases that convert oleic acid to many *trans* monenes, which then undergo reduction to stearic acid.

The enrichments were not different ($P > 0.05$) from zero for C16:0 or C18:2 (Fig. 2). The lack of ^{13}C-label in C16:0 rules out degradation of elaidic acid to shorter acyl chains or acetate and then utilization of the labeled acetate for elongation or even de novo synthesis of C16:0. It also rules out biohydrogenation of elaidic acid to C18:0 and then chain shortening of the C18:0 to C16:0. The lack of enrichment in C18:2 is consistent with the inability of anaerobes to synthesize polyunsaturated fatty acids. The synthesis of polyunsaturated fatty acids is restricted to aerobes via the oxygen-requiring desaturation of previously formed saturated fatty acids (10).

Unexpectedly, the enrichment data showed conversion of elaidic acid to two *cis* isomers, *cis*-9 and *cis*-11 C18:1. The interconversion of geometric isomers by isomerization has been demonstrated in several bacterial species. Usually, *cis* to *trans* isomerization is described more frequently than *trans* to *cis*. Bacterial species will convert oleic acid to elaidic acid to invoke changes in membrane permeability that protect them from a variety of growth inhibitors, including toxicants (10) or changes in ambient temperature (11). Kemp et al. (5) reported *cis/trans* isomerizations in both directions by a ruminal bacterium, although *cis* to *trans* was more extensive than *trans* to *cis*. The high initial concentration of elaidic acid in this study may have promoted abnormally high *trans* to *cis* isomerization. The percentage of oleic acid originating from elaidic acid can be estimated by dividing enrichment for oleic acid at 48 h ($6.4 \pm 0.87\%$) by the average for elaidic acid at 0 h ($36.4 \pm 1.51\%$) as described by Mosley et al. (3). This calculation reveals that 17.6% of the oleic acid in the cultures originated from elaidic acid and possible *trans* to *cis* isomerization. Other sources of oleic acid in the cultures were from the bacterial inoculum and the hay substrate.

It is possible to estimate the free energy and equilibrium constant for conversions of the *cis* double bonds to *trans* in hydrocarbons. For example, the free energy of formation of *cis*-2 hexene is 19.18 cal/mol and for *trans*-2 hexene it is 18.46 cal/mol (12). The difference in these energies is the free energy for the conversion of a *cis* double bond to a *trans* double bond and is 0.72 cal/mole. Thus, the *cis* double bond is slightly less stable as expected. The equilibrium constant for *cis* to *trans* isomerization estimated from the free energy at 25°C is 1.0012. If these hexene isomers, under the conditions given (perfect gases at 25°C), are reasonable models for the *cis* and *trans* double bonds in elaidic and oleic acids, the predicted equilibrium constant only imperceptibly favors the *trans* isomer with an equilibrium constant very close to one. The energies of positional isomerization of the double bonds for the monoenes are also expected to be quite small.

The possibility of *trans* to *cis* isomerization might partially account for the lower rates of oleic acid biohydrogenation that are often reported. Biohydrogenation was 10 to 25 percentage units lower for oleic acid than for linoleic acid in studies with sheep (13, 14) and cattle (15) fed a variety of fat sources. In rumen in vitro studies, rates of biohydrogenation also were lower for oleic acid compared with linoleic acid (16). However, if their data is adjusted for the 18% *trans* to *cis* isomerization observed in this study, oleic acid biohydrogenation increases about four to six percentage units, not enough to entirely make up the difference between oleic and linoleic acids.

Kemp et al. (5) pointed out that *cis/trans* isomerization may not all arise from enzymic activity. In their study, incubation of non-inoculated media for 24 h led to some *cis* to *trans* isomerization. The reverse was true in this study. Labeled elaidic was not found in oleic acid or any other fatty

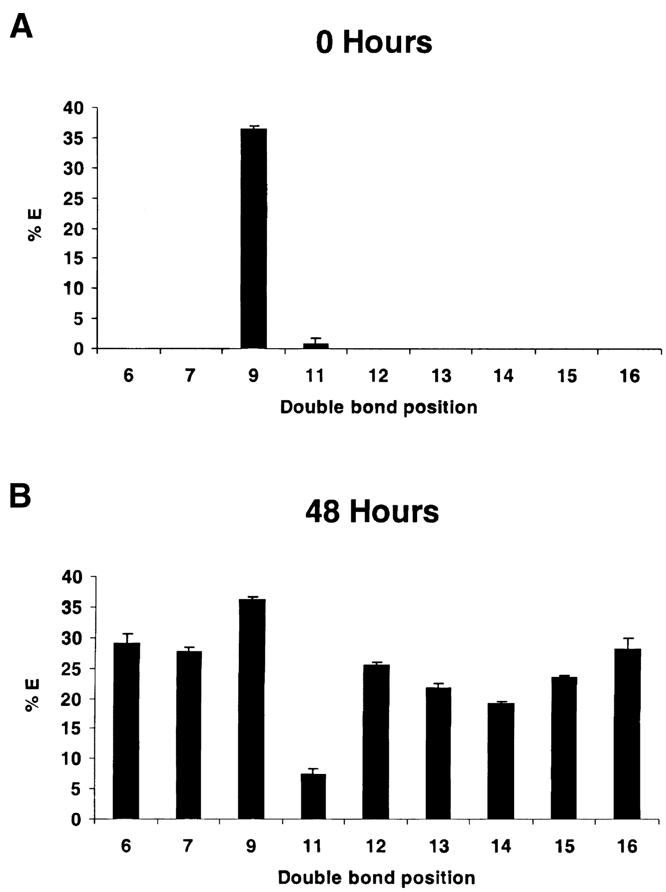

Fig. 3. The percentages of ^{13}C enrichment of *trans* C18:1 monoenes shown by double bond position at (A) 0 h and (B) 48 h of incubation time when a mixture (1:1) of elaidic and 18-^{13}C-elaidic acids were added (50 mg per culture) to cultures of mixed ruminal microbes. Each point is the mean of three replicates with standard deviations.

acid in the non-inoculated cultures. Thus, the presence of the bacterial inoculum was required for isomerization, which suggests an enzymatic process. However, the contribution of other non-protein compounds present in the inoculum (such as ions, vitamins, reducing agents, etc.) as the cause of isomerization cannot be ruled out.

Labeled elaidic acid also was converted to a number of other C18:1 *trans* positional isomers. At 0 h of incubation, enrichment was only detected in the elaidic acid added to the cultures (**Fig. 3**). At 12 h, enrichment was found in stearic acid (14%), *trans*-7 C18:1 (34%), *cis*-9 C18:1 (4%), *cis*-11 C18:1 (13%), elaidic acid (37%), and *trans*-11 C18:1 (6%). At 24 h, enrichment was found in stearic acid (17%), *cis*-9 C18:1 (5%), *cis*-11 C18:1 (9%), *trans*-6 C18:1 (29%), *trans*-7 C18:1 (33%), elaidic acid (38%), and *trans*-11 C18:1 (7%). Because enrichment for these isomers were similar for 12 h, 24 h, and 48 h, only 48 h enrichment data are shown in Fig. 3 for simplicity.

Incubation of 1-[^{13}C]oleate with mixed ruminal microbes also led to enrichment of a multitude of *trans*-C18:1 positional isomers (3). From these results, Mosley et al. (3) proposed the existence of one or more oleate isomerases that yield a multitude of *trans*-C18:1 isomers. The results of this study show that once a single *trans*-C18:1 isomer is formed, such as the elaidic acid, it can be converted to many other positional isomers. Therefore, biohydrogenation of oleic acid by ruminal microbes could produce a single *trans*-C18 isomer, such as *trans*-9 or *trans*-11 C18:1, followed by the subsequent isomerization of this isomer to many other positional isomers. Regardless of the pathway, the end result is the same: oleic acid is converted to a number of *trans*-C18:1 positional isomers when it is exposed to cultures of mixed ruminal microbes.

As information grows about the unique physiological functions of specific *trans* C18:1 isomers, it becomes more important to understand their origin. For instance, a portion of the *trans*-11 C18:1 isomer produced by ruminal microbes is converted to *cis*-9, *trans*-11 C18:2 by a tissue desaturase (17). The *cis*-9, *trans*-11 C18:2, commonly known as rumenic acid, has been identified as a potent anticarcinogen and modulator of body composition (18). Changes in composition of ruminant diets can alter the ratio of *trans* monoenes produced in the rumen. Increasing grain at the expense of high-fiber roughages shifts biohydrogenation toward increased *trans*-10 C18:1 at the expense of the *trans*-11 C18:1 isomer (2).

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