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 13C enrichment in component fatty acids by gas chromatography-mass spectrometry. At 0 h 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, 13C 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.

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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 13C-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 monoenes 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 13C-labeled elaidic acid to determine possible enrichment in stearic acid and other monene isomers.
MATERIALS AND METHODS

Reagents

Labelled elaidic acid (18-\[^{13}\text{C}\]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, 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 Georing 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-\[^{13}\text{C}\]elaidic acid and 50% unlabelled elaidic acid. Cultures containing unlabelled 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 flask 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\textsuperscript{®} 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 into 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 x 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 G fragments using the equation APE = (F + 1)/[F + (F + 1)]. In order to correct for the natural levels of 13\(^{\text{C}}\), the average APE of unlabeled cultures was subtracted from the APE of labeled cultures. Therefore, enrichment of the fatty acid with 13\(^{\text{C}}\) was calculated as (APE\(_{\text{labeled}}\) − average APE\(_{\text{unlabeled}}\))\times100.

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 cis9 C18:1 were small at 0 h and changed little.
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

![Graph A](image1)

![Graph B](image2)

**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.

**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 \(^{13}\text{C}\)-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

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**Fig. 3.** The percentages of \(^{13}\text{C}\) 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-\(^{13}\text{C}\)-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 (39%), 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-[13C]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 positional 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-C18:1 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). [11]

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REFERENCES