Kinetics of Ruminal Lipolysis of Triacylglycerol and Biohydrogenation of Long-Chain Fatty Acids: New Insights from Old Data

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ABSTRACT

Previous investigations into ruminal lipolysis of triacylglycerol and ruminal biohydrogenation (BH) of unsaturated long-chain fatty acids have generally quantified these processes with either zero-order or first-order kinetics. This investigation examined if Michaelis-Menten and other nonlinear kinetics might be useful for quantifying these processes. Data from 2 previously published *in vitro* experiments employing rumen fluid from sheep to investigate the lipolysis of trilinolein, the BH of *cis*-9, *cis*-12 linoleic acid (LA), and the BH of fatty acids derived from the lipolysis of trilinolein were used for the development of a multi-compartmental model. The model described the lipolysis of triacylglycerol well. The model also provided a good mathematical description of the resulting production of nonesterified fatty acids, the isomerization of nonesterified LA, and subsequent production of rumenic acid (RA), vaccenic acid (VA), and stearic acid (SA). However, the model described poorly the patterns of the concentrations of LA, RA, VA, and SA after incubation of trilinolein in rumen fluid. The model is consistent with known stoichiometry and biochemistry and is parsimonious in that it employs a minimal number of parameters to describe all of the major aspects of lipolysis and BH. The first step in the lipolysis of trilinolein was described by Michaelis-Menten kinetics ($V_{\max} = 529 \pm 16$ mg/L per h; $K_m = 698 \pm 41$ mg/L). Both subsequent lipolysis steps were approximated by a first-order (linear kinetics) rate constant ($k = 2.64 \pm 0.041$ /h). Isomerization of LA to RA was modeled by simple Michaelis-Menten kinetics ($V_{\max} = 2,421 \pm 83$ mg/L per h; $K_m = 440 \pm 22$ mg/L). The kinetics of the BH of RA to VA was described by a Michaelis-Menten-type process involving competitive inhibition by VA ($V_{\max} = 492 \pm 6.5$ mg/L per h; $K_m = 1$ mg/L). The final step, the BH of VA to SA, was modeled by a quasi-first-order process ($k = 0.533 \pm 0.021$ /h), but

as the concentration of VA increased, its BH appeared to be self-inhibited such that when the concentration of VA acid exceeded 517 ± 10.4 mg/L, BH was completely inhibited. The major new insights and benefits afforded by this model are 1) lipolysis and BH are described by nonlinear kinetics; 2) high concentrations of VA appear to inhibit its own BH; and 3) BH of RA appears to proceed at a much greater rate when triglyceride is present in the incubation medium. This model provides a conceptual framework for researching ruminal lipolysis and BH.

Key words: ruminal lipolysis, biohydrogenation, fatty acid, model

INTRODUCTION

Recently, Moate et al. (2004) presented a simple static model to describe ruminal lipolysis of dietary fats and ruminal biohydrogenation (BH) of long-chain fatty acids (LCFA). However, a better understanding of the nature and quantification of these dynamic kinetic processes may be needed if we are to make advances in developing sophisticated dynamic models that can predict with accuracy the pattern of individual LCFA absorbed from the intestine and subsequently the pattern of LCFA that appears in milk fat.

The kinetics of ruminal lipolysis and BH have been studied for many years (Reiser, 1951; Kepler et al., 1966). Since the early work of Garton et al. (1961), Kepler et al. (1966), and Hawke and Silcock (1969), it has been believed that only nonesterified unsaturated LCFA can be biohydrogenated in the rumen. Therefore, the fatty acids occurring on tri-, di-, or monoglycerides, and on monogalactosyl-diglyceride must necessarily be freed by lipolysis before they can be biohydrogenated. Noble et al. (1974) provided good evidence that the lipolysis of triglycerides (triacylglycerol, in modern terminology) takes place in a stepwise process. Triglyceride is hydrolyzed to produce a diglyceride and a NEFA. Next, the diglyceride is hydrolyzed to produce monoglyceride and a NEFA. Last, the monoglyceride is hydrolyzed to produce glycerol and a NEFA. While this

Received May 31, 2007.

Accepted October 9, 2007.

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stepwise process is taking place, liberated unsaturated NEFA can be biohydrogenated to more-saturated fatty acids.

The major pathway for the BH of free LA is as follows (Harfoot and Hazlewood, 1997): 1) linoleic acid (**LA**) is first isomerized to produce *cis*-9, *trans*-11 C18:2 or conjugated linoleic acid (**CLA**), also known as rumenic acid (**RA**); 2) RA is biohydrogenated to produce *trans*-11 C18:1 or vaccenic acid (**VA**); and 3) VA is biohydrogenated to produce C18:0 or stearic acid (**SA**).

All of the above LCFA are absorbed from the intestine, transferred to the mammary gland, and incorporated into milk fat. Recently, RA has been shown to have beneficial health and anticarcinogenic effects (Azain, 2003; Tricon et al., 2005). Not surprisingly, recent research has focused on elucidating how various dietary factors influence the rate of BH of LA. One problem associated with many previous in vivo and in vitro studies is that they have usually failed to separate the kinetics of lipolysis from BH and have generated rate constants that describe the "net" BH of certain unsaturated LCFA (Enjalbert et al., 2003; Troegeler-Meynadier et al., 2003; Ribeiro et al., 2007). Few studies have attempted to separately quantify the rate of lipolysis and the rates of BH.

A second issue related to lipolysis and BH is that few studies that have attempted to identify the specific nature of the kinetic processes involved. Most studies have simply assumed that the kinetics of lipolysis, BH, or lipolysis and BH combined can be described as simple first-order processes (Beam et al., 2000; Boufaied et al., 2003; Ribeiro et al., 2007). Lipolysis, isomerization, and BH are known to take place due to the action of lipases, isomerases, and reductases, respectively (Kepler et al., 1970; Harfoot and Hazlewood, 1997). Thus, instead of the commonly used first-order kinetics (linear kinetics) or zero-order kinetics (a specific form of nonlinear kinetics), it would seem likely that other nonlinear kinetics such as Michaelis-Menten enzyme kinetics may be a more appropriate way to analyze lipolysis and BH-related data.

The aim of the work described here was to investigate the nature of the separate kinetic processes describing lipolysis and BH and to estimate the magnitude of the rate constants describing these processes.

MATERIALS AND METHODS

Data

The data used in this investigation have been previously published and came from 2 experiments that aimed to elucidate the pathways involved in lipolysis and BH (Noble et al., 1974). Full details of the experimental procedures have been described by Noble et al.

(1974), but they did not model the data or estimate specific rate constants for lipolysis or BH.

Lipolysis

The data used to estimate rates of lipolysis were extracted from Table 2 on page 103 of the article by Noble et al. (1974). The lipolysis data came from an experiment in which 3 doses of trilinolein (460, 900, and 1,330 mg/L) were incubated in sheep rumen fluid in vitro. These doses of trilinolein correspond to LA doses of approximately 323, 646, and 970 mg/L. Rumen fluid was sampled from the flasks at 0, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0, and 7.0 h after the start of the incubation. The samples of rumen fluid were analyzed for triglyceride, "partial glycerides," and NEFA. Note that the term "partial glycerides" was coined by Noble et al. (1974) to represent the sum of mono- and diglyceride fatty acids.

Because lipolysis is a stepwise process, a compartmental model was developed to describe the process (Figure 1), and the sequential nature of the lipolysis process was described by the following set of differential equations and ancillary system equations:

$$\frac{dTG}{dt} = \frac{-k_1 TG(t) \{e^{-k_3 t}\}}{\{k_2 + TG(t)\}} \quad [1]$$

$$\frac{dDG}{dt} = \frac{2k_1 TG(t) \{e^{-k_3 t}\}}{3\{k_2 + TG(t)\}} - k_4 DG(t) \quad [2]$$

$$\frac{dMG}{dt} = \frac{k_4 DG(t)}{2} - k_4 MG(t) \quad [3]$$

$$\frac{dFFA}{dt} = \frac{k_1 TG(t) \{e^{-k_3 t}\}}{3\{k_2 + TG(t)\}} + \frac{k_4 DG(t)}{2} + k_4 MG(t) \quad [4]$$

$$PG(t) = MG(t) + DG(t) \quad [5]$$

$$TG(0) = \text{Dose}, DG(0) = 0, MG(0) = 0, FFA(0) = 0,$$

where TG (t) represents the concentration (mg/L) of triglyceride fatty acids present in the flask at time *t* hours after the start of the incubation; MG(t), DG(t), PG(t), and FFA(t) represent the concentrations of mono-glyceride fatty acids, diglyceride fatty acids, partial glycerides fatty acids, and NEFA, respectively. The constant *k*₁ (mg/L per h) is a Michaelis-Menten constant representing the V_{max} of the initial reaction, and *k*₂ (mg/L) is a Michaelis-Menten affinity constant. The term $\{e^{-k_3 t}\}$ in [1] is a unitless proportion used to reduce the V_{max} of the initial step in lipolysis as the age or duration of the incubation proceeds, and the constant *k*₃ has units of /h. The constant *k*₄ (/h) represents a first-

Figure 1. A schematic of the stepwise nonlinear model used to describe lipolysis of trilinolein. Partial glycerides represent the sum of diglyceride and monoglyceride fatty acids.

order fractional rate constant describing the lipolysis of both the di- and monoglycerides. The above compartmental model conserves matter and imposes known stoichiometry on the kinetic processes. In developing this model, the principal considerations were that the model had to be consistent with biology, the model had to be able to describe the experimental data, and the model had to be parsimonious; that is, use a simple structure and the least possible number of adjustable variables.

Biohydrogenation

Data for the investigation of the kinetic processes involved in BH of NEFA were extracted from Table 1 on page 102 of the article by Noble et al. (1974). These

data were from an experiment in which 3 doses of LA (320, 650, and 970 mg/L) were incubated in sheep rumen fluid in vitro. As in the previous experiment, rumen fluid was sampled from the flasks at 0, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0, and 7.0 h after the start of the incubation.

A compartmental model to describe the BH of the administered LA (as NEFA) was developed using standard modeling procedures (Wastney et al., 1999). A schematic of the model is shown in Figure 2. The equations used to model the initial isomerization and then BH of subsequent NEFA are as follows:

$$\frac{dLA}{dt} = \frac{-k_5 LA(t)}{\{k_6 + LA(t)\}} \quad [6]$$

$$\frac{dRA}{dt} = \frac{k_5 LA(t)}{\{k_6 + LA(t)\}} - \frac{k_7 RA(t)}{\{k_8 + RA(t) + VA(t)\}} \quad [7]$$

Figure 2. A schematic of the model used to describe the isomerization of linoleic acid (LA), followed by the stepwise biohydrogenation of rumenic acid (RA) to vaccenic acid (VA), and then the final biohydrogenation step to stearic acid (SA).

$$\frac{dVA}{dt} = \frac{k_7 RA(t)}{\{k_8 + RA(t) + VA(t)\}} - \left\{ k_9 VA(t) \frac{V(t)}{k_{10}} \right\} \quad [8]$$

$$\frac{dSA}{dt} = \frac{k_9 VA(t)V(t)}{k_{10}} \quad [9]$$

$$LA(0) = Dose, RA(0) = 0, VA(0) = 0, SA(0) = 0$$

$$V(t) = k_{10} - VA(t) \text{ if } VA(t) < k_{10}, \text{ else zero,}$$

where $LA(t)$, $RA(t)$, $VA(t)$, and $SA(t)$ are the concentrations (mg/L) of LA, RA, VA, and SA respectively at t hours after the start of the incubation. $Dose$ is the initial concentration of nonesterified LA. The Michaelis-Menten V_{max} constants k_5 and k_7 have units of mg/L per h, whereas k_6 and k_8 are Michaelis-Menten affinity constants with units of mg/L. In [8] and [9], k_9 (/h) is a first-order rate constant, whereas k_{10} is an inhibition constant with units of mg/L. When $VA(t)$ is very small with respect to k_{10} , then VA is biohydrogenated to SA in a quasi first-order process. However, as the concentration of VA increases, it inhibits its own BH such that when VA equals or exceeds k_{10} , the BH of VA is completely inhibited.

Biohydrogenation of Fatty Acids Derived from the Lipolysis of Trilinolein

Noble et al. (1974) showed that the time course profiles of fatty acids are quite different when trilinolein is lipolysed, in contrast to what happens when nonesterified LA is biohydrogenated. This brings up some questions. First, are the time course profiles of LA, RA, VA, and SA that are derived from the hydrolysis of trilinolein consistent with the rate constants that we have determined for the lipolysis of trilinolein and the BH of nonesterified LA, RA, VA, and SA? Or, do we need to hypothesize, as suggested by Noble et al. (1974), that the “hydrogenation of free linoleic acid may follow a pathway different from that of trilinolein-derived linoleic acid”?

In attempting to answer these questions we used all 10 rate constants estimated for lipolysis and BH and simulated (predicted) the concentrations of LA, RA, VA, and SA derived from the hydrolysis of trilinolein. The data for this investigation were extracted from Table 3 on page 104 of the article by Noble et al. (1974). The lipolysis of trilinolein is expected to release LA, and the time course for the concentration of LA released from trilinolein should be described by a combination of [4] and [6]:

$$\frac{dLA}{dt} = \frac{k_1 TG(t)\{e^{-k_3 t}\}}{3\{k_2 + TG(t)\}} + \frac{k_4 DG(t)}{2} + k_4 MG(t) - \frac{k_5 LA(t)}{\{k_6 + LA(t)\}} \quad [10]$$

$$TG(0) = Dose, LA(0) = 0,$$

where $Dose$ in this case is the initial concentration of LA esterified in TG. The concentrations of RA, VA, and SA are then described by equations [7], [8], and [9], respectively. Equation [10] involves only the assump-

tions that are imbedded in the development of equations [1] through [9].

Model Fitting and Data Analysis

The above equations to describe lipolysis (equations [1] to [4]) and BH (equations [5] to [10]) were implemented using WinSAAM (which can be downloaded from <http://www.winsaam.org>). WinSAAM, as described previously, is ideally suited for modeling nonlinear biological systems, especially enzyme kinetics (Wastney et al., 1999). The model for lipolysis (equations [1] to [4]) was simultaneously fitted to the data describing the 3 doses of triglyceride and metabolites. The model for BH (equations [5] to [9]) was simultaneously fitted to the data describing the 3 doses of LA and metabolites. The methods for using WinSAAM to fit models to data and to make predictions based on models (simulation) have been described by Stefanovski et al. (2003).

RESULTS AND DISCUSSION

Lipolysis

The model shown in Figure 1 was the simplest that we could devise that was consistent with biology and stoichiometry and that could describe the disappearance of triglyceride fatty acids and the appearance of fatty acids in partial glycerides and as NEFA. The data and model predictions for the disappearance of triglyceride fatty acids and appearance of partial glyceride fatty acids and NEFA are shown in Figure 3. In Figure 3A, the pattern of the disappearance of triglyceride fatty acids exhibits a “hockey-stick” shape, which typifies processes that can be described by Michaelis-Menten kinetics (Wastney et al., 1999). The model predictions shown in Figure 3A closely match the observed data. The least squares estimates ($\pm$ SD) of lipolysis parameters (k_1 , k_2 , k_3 , and k_4) are shown in Table 1. The initial step of hydrolysis of trilinolenin (described by equation [1]) had a Vmax of 529 ± 16 mg/L per h and a Km of 698 ± 41 mg/L. We have not been able to find in the scientific literature other estimates for Michaelis-Menten lipolysis parameters to compare with our parameters. However, our estimates of Vmax and Km for lipolysis are consistent with the in vitro zero-order lipolysis rates of between 200 and 400 mg/L per h reported by Gerson et al. (1985).

Beam et al. (2000) carried out an in vitro investigation in which soybean oil was added to rumen fluid at approximately 2, 4, 6, 8, and 10%. Inserting these “added dose” concentrations into equation [1] to obtain the initial rate of lipolysis, dividing by the added dose, and then multiplying by 100, we can compare the actual

Figure 3. In vitro disappearance of triglyceride fatty acids (top panel) from incubation media when 3 doses of trilinolenin (Δ , 460 mg/L; $\blacksquare$, 900 mg/L; $\bullet$, 1,330 mg/L) were incubated in rumen fluid from sheep. Panel A depicts triglyceride fatty acids, panel B depicts the appearance of “partial glycerides” (i.e., fatty acids in mono- and diglycerides), and panel C depicts the appearance of NEFA. The symbols represent data and the lines show the model predictions. Note that the concentration axes are scaled differently to better display the data.

Table 1. Parameters describing the in vitro lipolysis of trilinolein and biohydrogenation of unsaturated long-chain fatty acids

Parameter (unit)	Parameter explanation	Estimate $\pm$ SD
k_1 (mg/L/h)	Vmax for initial lipolysis step	529 $\pm$ 16
k_2 (mg/L)	Km for initial lipolysis step	698 $\pm$ 41
k_3 (/h)	A constant used to account for the ageing of the incubation mixture	0.0589 $\pm$ 0.0076
k_4 (/h)	First-order rate constant describing lipolysis of both the mono- and diglycerides	2.64 $\pm$ 0.041
k_5 (mg/L/h)	Vmax for isomerization of linoleic acid	2,421 $\pm$ 83
k_6 (mg/L)	Km for isomerization of linoleic acid	440 $\pm$ 22
k_7 (mg/L/h)	Vmax for biohydrogenation of rumenic acid	492 $\pm$ 6.5
k_8 (mg/L)	Km for biohydrogenation of rumenic acid (k_8 is nonadjustable)	1
k_9 (/h)	Quasi first-order rate constant describing biohydrogenation of vaccenic acid	0.533 $\pm$ 0.021
k_{10} (mg/L)	Vaccenic acid biohydrogenation self inhibition constant	517 $\pm$ 10.4

reported percentage/hour rates of lipolysis with percentage/hour lipolysis rates predicted from our model. The reported and predicted lipolysis rates (%/h) were: 41.4 vs. 59.4; 33 vs. 48.8; 25 vs. 41.4; 27 vs. 36.0; and 22.6 vs. 31.9 for 2, 4, 6, 8, and 10% added oil, respectively. An important finding by Beam et al. (2000) was that the initial percentage rate of lipolysis declined as the amount of added fat in the incubation medium increased, and our model predicts the same general trend. We note there are considerable systematic discrepancies in the magnitudes of the actual and predicted percentage/hour rates of lipolysis. However, these predicted in vitro lipolysis rates (%/h) for soybean oil are similar to the in vivo lipolysis rate of 52%/h that Moate et al. (2004) estimated for sunflower oil. This example demonstrates the difficulties in comparing rates of lipolysis and BH in different published studies.

Garton et al. (1961) found that the extent of lipolysis ranged from a high of 95% for linseed oil to a low of 40% for cocoa butter and Hawke and Robertson (1964) attributed the reduced extent of lipolysis to the presence of saturated fatty acids in the cocoa butter. Palmquist and Kinsey (1994) carried out in vitro lipolysis studies and reported that at concentrations above 2.5 g/L, lipolysis of free fats by ruminal microorganisms is zero-order with respect to substrate concentration. They reported zero-order lipolysis rates of 225, 46.2, 44.6, 40.4, and 12 mg/L per h for animal-vegetable blend, fish oil, palm oil, tallow, and hydrogenated tallow, respectively. Thus, the findings of Garton et al. (1961) and Palmquist and Kinsey (1994) demonstrate conclusively that fat type has a large effect on lipolysis rates. The soybean oil used by Beam et al. (2000) contained 11% palmitic acid and 4% SA, and so the rate (%/h) of lipolysis of soybean oil would be expected to be somewhat less than the predictions based on the lipolysis of trilinolein. Another issue that confounds comparisons is that in the published work about in vitro rates of lipolysis and BH, Noble et al. (1974), Gerson et al. (1985), Beam et al. (2000), and many other researchers have reported concentrations of added fat to

the incubation media and not reported the actual total triglyceride or NEFA concentrations present in the incubation media. Our model assumes that the triglyceride and NEFA concentrations are absolute concentrations. However, because the experimental data reported by Noble et al. (1974) are based on added or net concentrations of triglyceride and NEFA, our estimates of Vmax and Km for lipolysis and for biohydrogenation will necessarily be underestimates. With this in mind, a recommendation arising from this analysis is that in future in vitro investigations, researchers are encouraged to report the total concentrations of triglyceride and NEFA in the incubation media.

In equation [1], the term $\{\exp(-k_3 t)\}$ was used as a unitless proportion to reduce the Vmax as the age of the incubation increased. Thus, because k_3 was estimated as 0.0589 ± 0.0076 /h, we estimate that the maximal rate of lipolysis declined by approximately 34% by the conclusion of the incubation at 7 h. It is not clear what may have caused this decline in the maximal rate of lipolysis. The rate of lipolysis has been shown to be reduced when rumen fluid pH declines below 6.0 (Van Nevel and Demeyer, 1996). Noble et al. (1974) reported that the initial pH in their incubation vessels was 7.2, but they did not report the extent of pH change during the time course of their incubations. However, in closed in vitro systems, pH has been reported to decline by up to 0.6 pH units over a period of 7 h (Troegeler-Meynadier et al., 2003; Ribeiro et al., 2007). Thus, it is unlikely that pH was the major cause. Nevertheless, we speculate that the build-up in the incubation vessels of some by-product of fermentation (besides acid conditions) or a build-up of products of BH may have been responsible for this decline in the maximal rate of lipolysis. Alternatively, death of bacterial cells, depletion of lipase or of necessary cofactors, or depletion of feed particles (sites for lipolysis) during the incubation may also account for the slowing of the rate of lipolysis as the incubation progressed. In this regard, Gerson et al. (1985) showed that the rate of lipolysis declined from 400 to 200 mg/L per h when the NDF percentage declined from 42.8

to 19.5. Thus, there are many correlated factors, each of which might influence lipolysis, and it is difficult to determine specific causes of effects on lipolysis, especially in situations in which there may also be interactions.

The appearance of partial glyceride fatty acids in the incubation medium was described by equations [2], [3], and [5]. As can be seen from Figure 3B, the model predictions for the partial glyceride fatty acids did not closely match the data. This was surprising especially considering that the model predictions for NEFA (Figure 3C) closely matched the data, and the model predictions for NEFA are dependent on the predictions for the partial glyceride fatty acids. In attempting to modify equations to predict the transient existence of the partial glyceride fatty acids and the formation of NEFA, we examined a number of alternate kinetic forms including zero-order kinetics, Michaelis-Menten kinetics, and separate first-order rate constants to describe the hydrolysis of di- and monoglycerides. None of these alternate kinetic descriptions could give a better description of the partial glyceride fatty acids. We suspect that a possible explanation for the relatively poor fit of the model to the partial glyceride fatty acids but good fit to the NEFA data may be that the measurement of NEFA is technically simple and therefore likely to be more accurate than the measurement of partial glyceride fatty acids. In support of this inference, we note that Garton et al. (1961), using similar chromatographic procedures to those used by Noble et al. (1974), were unable to detect any partial glycerides during the lipolysis of linseed oil. In this model of lipolysis, the fact that k_1 , the Vmax for the initial lipolysis step, was 529 ± 16 mg/L per h, and k_2 , the Km, was 698 ± 41 mg/L per h, whereas k_4 , the first-order rate constant used to describe the lipolysis of the di- and monoglycerides, had a relatively high value of 2.64 ± 0.041 /h, confirms that the first step in the lipolysis of triglyceride is the overall rate-limiting process for lipolysis. Because k_4 was used to describe a first-order process and because k_4 had such a high value, this suggests that the enzyme(s) responsible for the lipolysis of the di- and monoglycerides was not saturated by substrate.

Houde et al. (2004) claim that "Lipolysis occurs at the substrate/water interface and therefore cannot be described by the Michaelis-Menten model, which is valid only for biocatalysis in a homogeneous phase, in which the substrate and the enzyme are soluble." We consider that all models are just approximations of reality, and we reject the strictly theoretical statement of Houde et al. (2004) because we have shown that Michaelis-Menten kinetics can, in fact, describe lipolysis. We consider that far from being inappropriate, Michaelis-Menten kinetics are especially suited for describing lipolysis

because at low substrate concentrations, they approximate to first-order kinetics, whereas at high substrate concentrations, they approximate to zero-order kinetics (Dixon and Webb, 1979). Thus, Michaelis-Menten kinetics can describe the lipolysis process over a range of substrate concentrations, especially in the concentration range between 0.25 and 2.5 g/L (which spans the range of triglyceride concentrations normally present in rumen fluid) and it is in this concentration range that lipolysis is not well described by either zero-order (Palmquist and Kinsey, 1994) or first-order kinetics.

Biohydrogenation

The data and model predictions of the disappearance of nonesterified LA from incubation medium and appearance of RA, VA, and SA are shown in Figure 4, panels A, B, C, and D, respectively. The least squares estimates ($\pm$ SD) of BH parameters (k_5 , k_6 , k_7 , k_9 , and k_{10}) are shown in Table 1. In Figure 4A, the hockey-stick shape to the curves depicting the disappearance of LA from rumen fluid is evidence for Michaelis-Menten type kinetics and the reasoning behind the development of equation [6]. The Vmax for the BH of LA was estimated to be $2,421 \pm 83$ mg/L per h, whereas the Km for this BH was 440 ± 22 mg/L. As with lipolysis, we have been unable to find comparable Michaelis-Menten type parameters in the scientific literature to describe the isomerization of LA. However, Gerson et al. (1985) reported that the zero-order rate of BH of LA ranged between 247 and 516 mg/L per h, which is consistent with our estimates for the Vmax and Km for the isomerization reaction. In contrast, Troegeler-Meynadier et al. (2003) reported that when LA in the form of triglyceride was incubated in vitro, the flux of disappearance of linoleic acid ranged between 8 and 28 mg/L per h in one experiment, and between 69 and 209 mg/L per h in another experiment. We note that our estimate for the Vmax for the isomerization of LA is much greater than the Vmax for the initial lipolysis reaction. With this in mind, we conclude that the flux rates for the disappearance of LA reported by Troegeler-Meynadier et al. (2003) must necessarily reflect the relatively slow rate of lipolysis that preceded the isomerization reaction.

In Figure 4B, the pattern of the concentrations of RA is typical of a transient product. The BH of RA was difficult to model. We tried a number of kinetic schemes and found that neither first-order kinetics nor a simple Michaelis-Menten kinetic process could account for the terminal phase of the RA curves. It appeared as though, during the latter stages of the incubation, something was inhibiting the BH of both RA and VA. We therefore tried bisubstrate "ping-pong" inhibition kinetics and

Figure 4. In vitro biohydrogenation of linoleic acid and metabolites when 3 doses of nonesterified linoleic acid ($\blacktriangle$, 320 mg/L; $\blacksquare$, 650 mg/L; $\bullet$, 970 mg/L) were incubated in rumen fluid from sheep. Panel A = linoleic acid; panel B = rumenic acid; panel C = vaccenic acid; and panel D = stearic acid. The symbols represent data and the lines show the model predictions. Note that the concentration and time axes are scaled differently to better display the data.

standard inhibition kinetics (Wastney et al., 1999). In contemplating the BH of RA and VA, we considered the possibility that a single enzyme might be responsible for the BH of both of these NEFA. Thus, we envisaged molecules of RA and VA competing for the fleeting embrace of the reducing enzyme from group B bacteria (Harfoot and Hazlewood, 1997). Alternatively, high VA concentration may reflect a high abundance of group A bacteria and low availability of the VA reductase, whereas low VA may reflect high abundance of group B bacteria and high availability of the VA reductase (Harfoot and Hazlewood, 1997). With this in mind, equation [7] is our attempt at describing a Michaelis-Menten or competitive inhibition type of reaction. We discounted the possibility that inhibition of the BH of RA and VA during the later stages of the incubation could be caused by the build-up of acid conditions in the incubation medium, because Ribeiro et al. (2007) found in an in vitro investigation that there was no significant difference in the rate of BH of RA to VA in either strongly or weakly buffered rumen fluid.

It has long been recognized that food particles are the major site for BH of unsaturated fatty acids in the rumen (Harfoot et al., 1973, 1975). Therefore, it is also possible that as the incubation progressed, depletion of feed particles may have played a role in reducing the rate of the BH of RA. We considered that any one of a large number of rumen fermentation products or a specific product of BH could be responsible. However, in the investigation reported here, VA was the NEFA that attained the greatest concentrations during the terminal stages of the incubation and we proceeded on the assumption that a high concentration of VA might account for an inhibitory effect on the BH of RA. However, it is also possible that VA is just a proxy for some other rumen fermentation product that is produced in parallel with VA or as discussed above, the depletion of feed particles. In many enzyme systems described by Michaelis-Menten kinetics, the affinity constant can be difficult to estimate. In modeling the BH of RA, we found difficulty in obtaining an identified estimate for the affinity constant k_s in equation [7]. To circumvent

this problem we fixed the value of k_8 at 1 mg/L. Thus, our estimates for k_7 , k_9 , and k_{10} are dependent upon this contrivance.

One intriguing phenomenon about the BH of C18 fatty acids is that under some circumstances, the BH process is incomplete and there can be a build-up of VA in the rumen (Kalscheur et al., 1997). In the experiment of Noble et al. (1974), when the initial dose of LA was 970 mg/L, almost all of the LA was converted to VA (Figure 4C), and, as can be seen in Figure 4D, with the high dose of LA, the BH of VA to SA almost completely ceased after 3 h of incubation. This suggests that some factor(s) must inhibit the BH of VA to SA. The data of Kalscheur et al. (1997) and AbuGhazaleh et al. (2005) indicate that rumen fluid VA concentrations tend to be elevated when dietary fiber content is low and rumen pH is substantially below 6. However, we do not consider that low pH was likely to be responsible for the inhibition of the BH of VA in the experiment of Noble et al. (1974). In Noble's experiment, in contrast to the high-dose LA incubation, there was no sustained build-up of VA concentration with the low-dose LA incubation, yet Noble et al. (1974) reported that all incubation vessels exhibited similar pH profiles during the incubations. The initial pH of the incubation medium was 7.2, and, as mentioned above, it is unlikely the pH would have declined below pH 6.0 during the 7 h of the incubation. Furthermore, Ribeiro et al. (2007) working with an in vitro system with pH above 6.0, reported no significant difference in the rate of biohydrogenation of VA to SA in either strongly or weakly buffered rumen fluid.

Polan et al. (1964) investigated the BH of LA in vitro. They reported that "Low levels of linoleic acid present in the incubation mixture were readily hydrogenated to stearic acid, but, when higher levels of linoleic acid were used, no stearic acid was formed, although the proportion of monoenoic acid increased." These findings of Polan et al. (1964) are similar to the findings of Noble et al. (1974), who reported that reduced amounts of SA formed when high doses of LA were incubated. Polan et al. (1964) interpreted their findings as indicating that high levels of LA per se directly inhibit the BH of VA. Noble et al. (1974) suggested that some other factors may be responsible for the inhibition of the BH of VA because the inhibition persisted for some time after the concentration of LA substrate has been reduced to a negligible level.

Moate et al. (2004), when modeling the in vivo BH of transoctadecanoic acid (C18:1 *trans*, which mostly represented VA) and flow of C18:1 *trans* to the duodenum, successfully used an exponential function to decrease the BH rate (%/h) as the concentration of total rumen nonesterified LCFA increased. In this modeling exercise we have attempted to identify a specific fatty

acid that could account for the inhibition of the BH of VA. In our opinion, LA cannot account for the inhibition of the BH of VA to SA, because it is essentially absent from the incubation medium after 1 h (Figure 4A). During the terminal stages of the incubation, only VA was present at very high concentrations, and therefore we postulated that high concentrations of VA might inhibit its own BH. Substrate inhibition at high concentrations is a recognized phenomenon in enzyme kinetics (Ferdinand, 1966). We therefore modeled the BH of VA by employing the concentration of VA as an inhibitor of its own BH. In Figure 4C we see that the self-inhibition kinetics described by equation [8] could very well describe the VA concentration patterns resulting from the incubation of the 3 doses of LA. The concentrations of SA were also reasonably well predicted (Figure 4D).

Biohydrogenation of Fatty Acids Derived from Lipolysis of Trilinolein

The data and model predictions on the concentrations of LA, RA, VA, and SA derived from the hydrolysis of trilinolein are shown in Figure 5. Compared with the patterns of LA concentrations following incubation of the NEFA (Figure 4A), our model accurately predicted that only very low concentrations of LA would be present following the incubation of trilinolein (Figure 5A). It is generally considered that, with respect to ruminal lipid transformations, isomerization and BH can only take place when there is a free carboxyl group on a fatty acid molecule (Hawke and Silcock, 1969). Despite the widespread acceptance of this axiom, we have been able to find only scant evidence for this (Garton et al., 1961; Garton 1964; Patton and Kesler, 1967). If LA attached to tri-, di-, or monoglycerides could be isomerized to RA, then we would have expected lower concentrations of LA than predicted by our model. The fact that the measured concentrations of LA in Figure 5A are all slightly greater than the concentrations predicted by our model provides additional circumstantial evidence in support of the thesis that there is a requirement for a free carboxyl group for the isomerization of LA to RA to occur.

Our model predicts that the concentrations of RA should be much greater than the actual measured concentrations (Figure 5B). This discrepancy between the model predictions and the data suggests that when RA is derived from the lipolysis of trilinolein, the BH of RA proceeds at a much faster rate than when triglyceride is not present within the incubation medium. Triglyceride and fatty acids in the rumen fluid are mostly adsorbed to the surface of feed particles (Harfoot et al., 1974, 1975) and BH is known to take place on the surface of feed particles (Harfoot et al., 1973). We speculate that

Figure 5. In vitro biohydrogenation of nonesterified fatty acids derived from the lipolysis of 3 doses of trilinolein (▲, 460 mg/L; ■, 900 mg/L; ●, 1,330 mg/L) incubated in rumen fluid from sheep. Panel A = linoleic acid; panel B = rumenic acid; panel C = vaccenic acid; and panel D = stearic acid. The symbols represent data and the lines show the model predictions. Note that the concentration axes are scaled differently to better display the data.

in comparison to the situation in which only NEFA are adsorbed to the surface of feed particles, the presence of adsorbed triglyceride on the surface of feed particles might cause a different orientation of adsorbed nonesterified RA and this different orientation might be more favorable for the interaction between RA and the reductase.

In Figures 5C and 5D, the concentration profiles of VA and SA were relatively similar to those that occur when nonesterified LA is incubated (Figure 4C and 4D). Part of the explanation why the simulations (predictions) of the fatty acids derived from trilinolein shown in Figures 5A, 5B, 5C, and 5D do not closely match the observed data is that we have not attempted to model a number of fatty acid isomers that were only evident during the incubation of trilinolein. Difficulties encountered in modeling the BH of fatty acids derived from trilinolein may have been due to lack of accuracy in measurement of the very low concentrations of LA and

RA. The other issue that had a large effect on the modeling of both lipolysis and BH was the fact that data from only 8 time points were available.

Comparison with Other Models

No existing dynamic models describe the ruminal lipolysis of triacylglycerol as well as the stepwise BH of individual unsaturated LCFA. Baldwin et al. (1987) presented a dynamic model (Molly) that describes ruminal lipolysis of lipid and BH of unsaturated fatty acids. In Molly, all dietary lipid is assumed to contain unsaturated fatty acids that, on entering the rumen, is subjected to lipolysis. The resulting aggregate of unsaturated fatty acids can then either be reduced to saturated fatty acids and pass from the rumen, or the unsaturated fatty acids can be utilized for microbial growth. These processes are all described by simple first-order kinetics. Thus, with respect to ruminal lipolysis and BH, the

overly simplistic assumptions and level of aggregation in Molly limits the accuracy and scope of its predictions.

Dijkstra et al. (2000) have presented a qualitative description of a rumen fat model in which ruminal lipolysis of fat is depicted by means of a first-order rate constant and BH of an aggregate of unsaturated LCFA by a Michaelis-Menten equation. However, neither the equations nor parameter values of their model are presented, preventing comparison with the parameter values estimated in this work.

More recently, Ribeiro et al. (2007) presented a dynamic model of in vitro BH of unsaturated fatty acids in alfalfa. However, their model really depicts a combination of lipolysis and BH, because they did not model the separate kinetics of lipolysis and BH. Further, the model of Ribeiro et al. (2007) utilizes first-order (linear) kinetics to describe the net BH of individual unsaturated fatty acids. Thus, although the model of Ribeiro et al. (2007) could well describe the temporal patterns of LCFA intermediates following the incubation of fresh alfalfa and alfalfa hay with low concentrations of total fatty acids (2.1 and 0.8 %/DM, respectively), their model does not have nonlinear rate constants that would allow the accurate description of an accumulation of VA that might occur if an alfalfa substrate containing high concentrations of LA or linolenic acid were to be incubated.

CONCLUSIONS

We have presented a compartmental model to describe the in vitro (ruminal) lipolysis of triglyceride, the isomerization of nonesterified LA, and BH of RA and VA in rumen fluid from sheep. The model is consistent with stoichiometry and biochemistry and is parsimonious in that it employs a minimal number of parameters to describe all of the major aspects of lipolysis and BH. The model describes various reactions by first-order (linear kinetics) and by Michaelis-Menten and other nonlinear forms of kinetics. The estimates and standard deviations are provided for the 9 adjustable parameters concerned with lipolysis and BH. All but one of these parameters were well identified. The model described well the lipolysis of triacylglycerol and resulting production of NEFA. The model also described well the isomerization of nonesterified LA and subsequent production of RA, VA, and SA. However, the model poorly described the patterns of the concentrations of LA, RA, VA, and SA after incubation of trilinolein in sheep rumen fluid. An important insight gained from this modeling investigation is that the BH of VA may be self inhibited. Another important insight is that the rate of BH of RA may be greater in the presence of trilinolein than in the presence of NEFA only. It is considered that experiments investigating the potential for high

concentrations of VA to inhibit the BH of not only VA, but also RA are warranted. The modeling carried out here highlights the desire that when in vitro investigations of lipolysis and BH are being conducted researchers are encouraged to report the profiles of the absolute concentrations of triglycerides, esterified fatty acids, and NEFA in incubation media as well as pH and measures of feed particles and culture activity/viability at each time point. Furthermore, because the in vitro investigations modeled here were with sheep rumen fluid, further similar investigations with rumen fluid from cows are warranted.

ACKNOWLEDGMENTS

We are thankful to R. C. Noble, J. H. Moore, and C. G. Harfoot for publishing (Noble et al., 1974) data in a form that made possible the work presented in this paper.

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