Comparison of Techniques to Determine the Clearance of Ruminal Volatile Fatty Acids

J. C. Resende Júnior,*¹ M. N. Pereira,† H. Bôer,‡ and S. Tamminga‡

*Departamento de Medicina Veterinária, and

†Departamento de Zootecnia Universidade Federal de Lavras, Lavras, MG, Brazil, 37200-000

‡Wageningen University, Animal Sciences, Animal Nutrition Group, Wageningen, The Netherlands

ABSTRACT

The objective of this experiment was to compare measurements of fractional clearance rates obtained by using an unlabeled valerate-CoEDTA technique with measurements obtained by using a ¹³C-labeled volatile fatty acids (VFA) technique. The exponential decay rate of the ¹³C/¹²C ratio after pulse-dosing ¹³C-acetate, ¹³C-propionate, or ¹³C-butyrate into the rumen was compared with the decay rate of rumen valerate concentration following a simultaneous pulse dose. The unlabeled valerate, CoEDTA, and each labeled VFA, one at a time, were concurrently mixed with the evacuated ruminal content of 6 lactating cows in two 3 × 3 Latin squares. The clearance of VFA by passage to the omasum was assumed to be equivalent to the decay in ruminal Co concentration and was around 50% of the total clearance. Acetate, propionate, and butyrate had similar fractional clearance rates (31.2, 33.4, 30.4%/h, respectively), but propionate had a higher absorption rate (19.2%/h) than butyrate (14.2%/h). Linear regression determination coefficients using the valerate clearance rate as an estimator for acetate, propionate, and butyrate rumen clearance were 0.51, 0.56, and 0.99, respectively. In a second experiment, the ¹³C-valerate fractional clearance rate estimate (33.7%/h) was similar to the estimate obtained with unlabeled valerate (35.0%/h) by the valerate-Co technique. No ¹³C enrichment of rumen microbes was noted 4 h after the intraruminal infusion of ¹³C-valerate. Fractional VFA absorption rate estimates obtained in both techniques were similar, although both were lower than estimates reported in the literature by other methods.

Key words: volatile fatty acid, rumen, clearance rate, absorption

INTRODUCTION

Volatile fatty acids are produced in the gastrointestinal tract of ruminants as end products of microbial

metabolism and may provide up to 80% of the daily ME absorption (Bergman, 1990). The removal (clearance) of VFA from the reticulorumen occurs by 2 processes: absorption through the wall and passage with the rumen fluid through the reticulum-omasum orifice (Peters et al., 1990). If the VFA production rate exceeds the clearance rate, VFA will accumulate in the rumen. This phenomenon may excessively lower rumen content pH and cause the metabolic disturbance known as rumen acidosis (Barker et al., 1995). Rumen acidosis may have negative effects on animal performance and health, causing deleterious effects on feed intake (Elliot et al., 1995), rumen fiber degradation (Grant and Mertens, 1992), rumen motility (Crichlow and Chaplin, 1985), microbial production (Hoover, 1986), and rumen wall morphology (Jensen et al., 1954).

For the study and control of rumen acidosis, a technique capable of measuring VFA ruminal clearance by passage with the liquid phase and by rumen wall absorption is very useful. Various techniques have been used to determine VFA absorption rates, such as the collection of blood from the portal vein to measure VFA appearance (Huntington et al., 1983), the intraruminal infusion of VFA enriched with radioactive (Sutton et al., 2003) or stable isotopes (Nozière et al., 2000; Kristensen, 2001), the ruminal evacuation and introduction of a VFA solution into the ventral sac of the washed rumen (Dijkstra et al., 1993), and the continuous infusion of nonlabeled VFA in a nonevacuated rumen (Peters et al., 1990). Due to the rumen wall metabolism of VFA, the use of the portal appearance of VFA as an absorption measure has restrictions (Bergman, 1990; Gabel, 1995; Kristensen, 2001). Measurements that use radioactive isotopes are expensive and have undesirable risk factors to humans, animals, and the environment (ISO Analytical, 2002). Although stable isotopes present great potential, their use can be limited by both the technological apparatus and technique cost (ISO Analytical, 2002). Measurements done in an evacuated rumen may also generate absorption estimates in an atypical physiological condition, certainly with a different ratio of rumen absorptive surface area to VFA solu-

Received November 26, 2005.

Accepted January 31, 2006.

¹Corresponding author: joacrcj@ufla.br

tion than that found in the rumen of a productive animal.

The technique that uses stable carbon isotopes as a marker assumes that ^{13}C from the labeled VFA molecule will behave similarly to the ^{12}C from a similar VFA molecule, and both will similarly disappear from the rumen by both absorption and passage with the fluid phase. The mathematical constant that describes the ruminal content exponential decay of the $^{13}\text{C}/^{12}\text{C}$ ratio over time is an estimate of VFA disappearance, the fractional clearance rate (Chen et al., 1997).

The valerate-cobalt technique (**HVal-Co**) is an alternative technique to measure rumen VFA clearance, and is based on the use of valerate (**HVal**) as a marker (Allen et al., 2000). Valerate is a 5-carbon carboxylic acid naturally produced in the rumen environment (Gray et al., 1952); it occurs at a low concentration in the digesta, and is not significantly metabolized by rumen microorganisms (Allen et al., 2000). The HVal-Co technique is based on the exponential decay of the ruminal concentration of HVal after pulse dosing the marker into the rumen, and the fractional rate of clearance is corrected by the fractional rate of rumen fluid passage. Advantages of this technique are low cost, operational simplicity, and low invasiveness. Such a technique allows the estimation of full rumen VFA fractional clearance rates by passage and by absorption simultaneously, with the advantage of being executable in a large number of animals fed under productive nutritional levels.

The objective of this experiment was to compare measurements of VFA fractional clearance rates obtained by using the HVal-Co method to estimates obtained with a ^{13}C -labeled VFA method. Another objective was to evaluate the possibility of obtaining estimates of acetate (**HAc**), propionate (**HPr**), and butyrate (**HBu**) fractional absorption rates from data generated with the HVal-Co technique.

MATERIALS AND METHODS

Experiment 1

Six lactating, rumen-cannulated Holstein-Friesian cows were randomly allocated to two 3×3 Latin squares with 14-d periods. Treatments consisted of ruminal infusions of ^{13}C -labeled HAc, HPr, or HBu. Cows were from 20 to 125 DIM, the mean being 48, of their second or third lactation, and their mean BW was 568 kg. They were kept confined in a tie-stall barn at the Wageningen Institute of Animal Science (Wageningen University, The Netherlands) during the experiment, fed freshly cut perennial ryegrass *ad libitum*, and supplemented daily with 2.8 kg of concentrates supplied twice a day separately from the forage. Mean daily total and forage

DM intakes were 17.7 and 15.6 kg, respectively, and daily milk production was 25.7 kg.

Collection of data occurred on d 14 of each period. On this day cows did not receive the second daily portion of concentrates normally offered at 1500 h. The VFA infusion procedures started at 1900 h, following a complete evacuation of the ruminal content for digesta sampling, and thorough mixing of the marker solution. The manually evacuated digesta was transferred to an isothermal box. A composite rumen digesta sample was formed from 10 individual samples collected throughout the evacuation procedure for DM determination. After homogenization of the marker solution, rumen content weight was determined and the digesta were placed back into the rumen. The ruminal fluid volume was calculated based on these measurements. Animals were not fed during the rumen evacuation procedure or during the next 4 h of rumen sampling.

The 2.5-L marker solution contained 30 g of CoEDTA (Uden et al., 1980), 300 g of *n*-valeric acid (Merck, Darmstadt, Germany), and, according to each treatment, 500 mg of $1\text{-}^{13}\text{C}$ -Na-acetate, 600 mg of $1\text{-}^{13}\text{C}$ -Na-propionate, or 650 mg of $1\text{-}^{13}\text{C}$ -Na-butyrate, all 99% enriched with ^{13}C (Campro Scientific, Veenendaal, The Netherlands). The infused amount of labeled VFA was based on a theoretical estimate necessary to achieve ^{13}C -enrichment of the total ruminal VFA carbon of 1.1033%, assuming a 65:20:15 ratio of HAc, HPr, and HBu. Marker solution pH was adjusted to 6.5 with the addition of a small amount of 50% NaOH solution. Immediately before the rumen evacuation, a 100-mL sample of rumen fluid was obtained from the ventral sac using a perforated tube connected to a suction device to determine the ruminal basal $^{13}\text{C}/^{12}\text{C}$ ratio. Rumen samples were also obtained immediately after returning the evacuated ruminal content (time zero) and at 15, 30, 60, 120, and 240 min. Rumen sample pH was determined immediately at each sampling time. Mean pH for was the arithmetic mean of the values for the 6 samples collected during the 4-h sampling period. Samples were divided into 3 aliquots. A 70-mL aliquot used to determine ^{13}C concentration and a 10-mL aliquot for Co analysis were immediately frozen at -18°C ; the third aliquot (5 mL) used for VFA analysis was mixed with 250 μL of 85% orthophosphoric acid (Merck) just before being frozen at -18°C .

To determine the rumen content ^{13}C concentration, VFA were isolated by distillation. As described by Tóthi (2003), the thawed rumen fluid was centrifuged at $855 \times g$ for 10 min at room temperature. Forty grams of the supernatant was transferred with 35 mL of demineralized water to a Kjeldahl distillation flask. Distillation was then performed in the presence of Na_2SO_4 and 96% sulfuric acid. Four drops of concentrated phosphoric

acid were added to the distillate and demineralized water was added to the flask to achieve a 100-mL final volume. This solution was frozen until the determination of ^{13}C : ^{12}C ratios by isotope ratio mass spectrometry (IRMS) as described by Boutton (1991). However, the machine used cannot distinguish the specific VFA in which ^{13}C enrichment has occurred, so the results obtained refer to whole ^{13}C and whole ^{12}C independently of the molecule containing the atom.

The rumen fluid VFA concentration was determined in the supernatant obtained by centrifugation at $8,855 \times g$ for 10 min at room temperature. Samples were analyzed by GLC (Fisons Chromatograph HRGC Mega 2 GC with a flame-ionization detector GenTech Scientific, Inc., Arcade, NY). The chromatographic glass column had an i.d. of 2 mm, length of 1.83 m, and was filled with chromosorb 101, mesh 80/100 (Supelco, Bellefonte, PA). The carrier gas was N_2 saturated with 99% formic acid (Merck). Column oven temperature was 190°C ; the injector temperature was 186°C ; the flame-ionization detector temperature was 225°C ; and the carrier gas flow in the column was 35 mL/min. The mean VFA concentration was the arithmetic mean of the values for the 6 rumen samples collected during the 4-h sampling period.

For each rumen fluid sample obtained over time, the carbon concentration coming from each analyzed VFA was obtained by multiplying each VFA concentration (mM) by the respective number of carbons in that VFA molecule. The analyzed VFA were HAc, HPr, HBu, HVal, isobutyrate, and 2- and 3-methylbutyrate. The percentage of non-HVal carbon in the total VFA carbon pool was calculated. This calculation was performed because a large amount (300 g) of exogenous HVal had been introduced into the rumen fluid with the marker solution. Based on the calculated percentage on non-HVal carbon in each sample, the percentage of non-HVal carbon in the IRMS-measured total carbon pool was obtained. The ^{13}C pool (marker) in each sample was then divided by the pool of total non-HVal carbon minus the ^{13}C pool (marker). A ratio of ^{13}C coming from each VFA to non-HVal ^{12}C was obtained. This ratio from the ruminal fluid sample collected just before evacuating the rumen (basal) was subtracted from the ratio determined for each sampling time. The nonlinear regression coefficient of the exponential equation describing the decay of the $^{13}\text{C}/^{12}\text{C}$ ratio over time was used to represent the fractional rate of clearance for each labeled and infused VFA. A simple exponential equation describing the change in $^{13}\text{C}/^{12}\text{C}$ ratio (R) over time was used: $R_t = R_0 \times e^{-k \times t}$. Similarly, for the HVal-Co technique, the exponential decay rate in ruminal HVal concentration was used to estimate the fractional rate of HVal clearance.

The concentration of Co in each sample was determined in the supernatant obtained by centrifugation of rumen fluid at $1,924 \times g$ for 15 min. Cobalt concentration was measured by atomic absorption spectroscopy (SpectrAA 300, Varian, Middelburg, The Netherlands), as described by Uden et al. (1980), at a wavelength of 251.0 nm. The disappearance of ruminal VFA by passage with the fluid phase was assumed equivalent to the ruminal fluid fractional passage rate determined by the exponential decay rate in ruminal Co concentration over time. The fractional rates of absorption for each labeled VFA and for HVal were calculated by subtracting the fractional passage rate from the fractional clearance rates. Because the fractional rates of absorption were indirectly calculated, these include a sum of errors associated with fractional clearance and passage rate determinations. The rumen volume was estimated by extrapolating the equation describing the ruminal Co concentration at time zero and dividing this intercept value by the infused amount of Co.

Experiment 2

A marker solution identical to the one used in Experiment 1 was prepared with 5- ^{13}C -n-valerate. This solution was concurrently mixed with the ruminal contents of the same 6 cows managed under identical conditions as in Experiment 1. Cows were from 56 to 161 DIM, the mean being 86; daily milk production was 28.3 kg; and the mean BW was 553 kg. The objective was to compare the estimated ruminal fractional clearance rate of ^{13}C -labeled HVal to the estimate obtained with unlabeled HVal by the HVal-Co technique. The amount of 5- ^{13}C -n-valerate placed into the marker solution was 325 mg. Experimental procedures were identical to those described earlier.

With the aim of evaluating valerate metabolism by rumen microorganisms, the incorporation of ^{13}C from HVal into the rumen microbial mass was determined. A 500-mL sample of rumen fluid was collected just before rumen evacuation and another fluid sample was obtained 4 h after returning the evacuated digesta (inoculated with the marker solution) to the cow. These fluid samples were stored for about 14 h at 2°C . A microbial pellet was then isolated by serial centrifugation and filtration (Robinson et al., 1987). The pellet was freeze-dried for storage until analysis. The freeze-dried pellet was diluted in demineralized water and submitted to the $^{13}\text{C}/^{12}\text{C}$ analyses in the IRMS.

Statistical Analyses

In Experiment 1 the fractional rates of VFA clearance, absorption, and passage, the mean rumen VFA

Table 1. Dry matter intake, rumen VFA concentration, rumen acetate/propionate ratio, rumen mean pH, and rumen volume in Experiments 1 and 2¹

	Experiment 1					Experiment 2
	¹³ C-HAc	¹³ C-HPr	¹³ C-HBu	SE	P-value ²	¹³ C-Val
Total DM intake (kg/d)	18.1	17.8	17.2	0.3	0.13	17.9
Roughage DM intake (kg/d)	15.6	15.4	14.8	0.3	0.14	15.5
Total VFA (mM)	142.6	137.2	141.7	3.2	0.47	144.7
Acetate (mM)	80.0	77.4	79.8	1.9	0.58	79.9
Propionate (mM)	24.4	23.7	23.6	0.79	0.76	25.6
Isobutyrate (mM)	1.0	1.0	1.0	0.0	0.57	1.2
Butyrate (mM)	14.6	13.6	14.0	0.4	0.31	15.2
Methylbutyrate ³ (mM)	2.0	1.8	1.8	0.1	0.22	1.7
Valerate (mM)	20.1	19.7	21.5	0.7	0.29	21.2
Acetate/propionate	3.34	3.28	3.60	0.10	0.20	3.15
pH	6.03	6.02	6.09	0.05	0.63	6.11
Rumen volume (L)	93	94	89	3	0.43	88

¹¹³C-HAc, ¹³C-HPr, ¹³C-HBu, ¹³C-HVal = ¹³C-labeled acetate, propionate, butyrate, and valerate, respectively.

²Probability for acid effect.

³Sum of 3-methylbutyrate and 2-methylbutyrate.

concentration, the mean rumen pH, and both measures of rumen fluid volume were analyzed with the GLM procedure of SAS (SAS Institute, 1999), according to the following model:

$$Y_{ijkl} = \mu + S_i + C_{j(i)} + P_k + V_l + e_{ijkl}$$

where μ = overall mean; S_i = square effect ($i = 1$ or 2); $C_{j(i)}$ = cow within square effect ($j = 1$ to 6); P_k = period effect ($k = 1$ to 3); V_l = labeled VFA effect ($l = \text{HAc, HPr, or HBu}$); and e_{ijkl} = residual error, assumed independently and identically distributed in a normal distribution with mean zero and variance σ^2 .

Linear regressions between the fractional rates of clearance of unlabeled HVal and the rates of ¹³C-labeled HAc, HPr, and HBr were performed.

In Experiment 2, the fractional rates of HVal clearance, absorption, and passage estimated with the labeled VFA technique and the HVal-Co technique were compared with the following model:

$$Y_{ij} = \mu + C_i + T_j + e_{ij}$$

where μ = overall mean; C_i = cow effect ($j = 1$ to 6); T_j = HVal technique effect ($i = ^{13}\text{C}$ or HVal-Co); and e_{ij} = residual error, assumed independently and identically distributed in a normal distribution with mean zero and variance σ^2 .

The fractional rates of clearance of ¹³C-HVal were regressed against the fractional rates determined with the HVal-Co technique. Microbial mass ¹³C enrichment before and after ruminal content inoculation with labeled HVal was evaluated with the same model by exchanging the "HVal technique effect" by an "infusion time effect" (before or after inoculation).

RESULTS AND DISCUSSION

Both the HAc/HPr ratio and rumen pH (Table 1) were typical for animals that consume diets with a high proportion of forage and have a low probability of developing disturbances related to rumen acidosis (Pereira and Armentano, 2000), showing that these data are consistent with normally functioning rumen fermentation.

Rumen evacuation seems to be a major disturbance of normal rumen function. It interrupts VFA absorption and rumen passage and it destroys stratification. The technique used in this research was such that the entire procedure took about 15 min, and the rumen contents were kept in an insulated container outside the cow. Visual inspection of the values for pH and shift in pH between the moment of rumen evacuation and the time before and after were in the same range as those between the other sampling times. Bailable liquids were 5% below the total rumen content, on average. Hence, disturbance of the layers in the rumen was minimal. With all these precautions the evacuation procedure had only a minimal disturbing effect on rumen function. Some authors have purposed the addition of markers without rumen evacuation (Allen et al., 2000; Sutton et al., 2003). However, when rumen evacuation is not used, the ruminal environment can also suffer disturbance, because a good mix mechanism is necessary. Moreover, without rumen evacuation the necessary volume of marker solution may be very high (Allen et al., 2000) or sometimes there is problem with the fluid marker curve, probably because of a bad mixture not permitting a good ruminal volume estimate (Sutton et al., 2003). The purpose of the current experiment was just to compare the absorption and passage rate. The

minimal disturbance in ruminal environment was applied to all cows and all treatments so it probably did not interfere in this comparison. Thus, in the current experiment, good mixing of the markers with ruminal digesta obtained by rumen evacuation was more important than ruminal environment disturbance on comparison of clearance rates.

Because of the addition of 300 g of HVal immediately before sampling time zero, the rumen concentration of HVal was higher than that of HBu (Table 1), which is considered physiologically abnormal (Bergman, 1990). However, the addition of this amount of HVal was not enough to induce either the rumen pH nonphysiological values or the other VFA molar proportions. The VFA rumen concentration immediately before the rumen evacuation in experiment 1 was (mM): 86.8 for HAc, 27.4 for HPr, 15.0 for HBu, 1.1 for i-HBu, 1.9 for m-Hbu, and 1.7 for HVal. In Experiment 2, these values were 85.0, 28.0, 16.0, 1.2, 1.6, and 1.5, respectively. The addition of HVal had no effect on the molar ratios of the other VFA at the collection times ($P > 0.58$ in Experiment 1 and $P > 0.15$ in Experiment 2). Therefore, the conversion of added HVal to other VFA does not seem to have been a priority route for the disappearance of this marker. Although the conversion of C from HAc and HPr to HVal is possible (Gray et al., 1952; Kristensen, 2001), no published data showing the conversion of rumen HVal to another VFA have been found. No effect of the intraruminal excess of HVal on the health and performance of the animals was observed in any of the 24 infusions carried out in this work, demonstrating that the technique of HVal-Co is biologically safe. However, this was an empirical observation based solely on animal inspection and on absence of perceptive disease. Parameters such as blood acid/base equilibrium, respiration rate, heart rate, and other measures of distress were not evaluated.

The $^{13}\text{C}/^{12}\text{C}$ ratio of the microbial mass collected before the introduction of labeled HVal was higher than that collected 4 h after the addition of the markers (Figure 1). The reduction in the $^{13}\text{C}/^{12}\text{C}$ ratio between the 2 collection times can be related to the alimentary handling. At the time of the fluid collection (before the rumen evacuation, 1900 h), at least 13 h had passed since the last feeding of concentrates to the animals (provided at 0600 h). At the time of the fluid collection, 4 h after time zero, more than 17 h had passed since the last feeding of concentrates to the animals. The concentrate used was based on components derived from C_4 plants, which have higher ^{13}C concentration than C_3 plants (Yeh and Wang, 2001). It is probable that the $^{13}\text{C}/^{12}\text{C}$ ratio in the microbial mass had fallen because of the substitution of the substrate used for microbial growth. Therefore, the data obtained here do

Figure 1. Ratio of $^{13}\text{C}/^{12}\text{C}$ in the microbial mass isolated from the rumen fluid of lactating cows before rumen evacuation (white bar) and 4 h after returning to the cow the digesta inoculated with ^{13}C -labeled valerate (black bar). $P < 0.01$ (Experiment 2).

not indicate if the disappearance of ^{13}C -Hval due to its incorporation in microbial mass could be significant, because the substrate effect discussed above could obscure an eventual incorporation effect. Allen et al. (2000) found that the calculated decline rate in valerate concentration after incubation in vitro with ruminal microorganisms for 24 h was 0.003/h, which is small relative to the clearance rate and should have little effect on the accuracy of the calculated absorption rate.

The fractional rate of rumen wall absorption of ^{13}C , introduced in the rumen as HBu, was the lowest of the 3 VFA (Table 2). The fractional clearance rates of labeled carbon introduced in the rumen as HAc, HPr, or HBu was around 30%/h (Table 2) and the rates were not proportional to the size of the carbon chain of each acid. Gabel (1995) stated that at physiological pH, the absorption rate of butyrate is about 1.35 times higher than that of propionate and the absorption rate of propionate is 1.11 to 1.41 times higher than that of acetate, but Gabel recognized that there are factors involved in VFA absorption other than carbon chain length. In the current study, the propionate absorption rate was 1.25 times higher than acetate in agreement with Gabel (1995), but one inversion occurred because the propionate absorption rate was 1.35 times higher than butyrate.

A possible explanation for the similarity in the clearance rates between acids (Table 2) is the carbon 1 inter-conversion between HAc, HPr, and HBu (Sutton et al., 2003). The $^{13}\text{C}/^{12}\text{C}$ ratio decrease measured in this work represents all the stable isotopes from VFA in the rumen environment because the technique used did not separate each labeled VFA before the mass spectrometry measurement had been done. The similarity in the fractional rates might be due to the disappearance of ^{13}C introduced in rumen as 1- ^{13}C -VFA. It does not, however,

Table 2. Fractional VFA¹ rumen clearance rate (kc), fractional passage rate to omasum with liquid phase (kp), and fractional rumen wall absorption rate (ka) estimated by labeled VFA in Experiment 1 and estimated by labeled valerate and unlabeled valerate in the same animals simultaneously in Experiment 2

Rate	Experiment 1					Experiment 2			
	¹³ C-HAc	¹³ C-HPr	¹³ C-HBu	SEM	P ²	¹³ C-HVal	HVal-Co	SEM	P ³
Kc ⁴ (%/h)	31.2	33.4	30.4	1.15	0.21	33.7	35.0	1.3	0.53
Ka ⁵ (%/h)	15.4	19.2	14.2	0.96	0.02	18.7	19.9	1.3	0.53
Kp ⁶ (%/h)	15.7	14.2	16.1	0.77	0.23	15.1			

¹ ¹³C-HAc, ¹³C-HPr, ¹³C-HBu, ¹³C-HVal = labeled acetate, propionate, butyrate and valerate, respectively; HVal-Co = unlabeled valerate.

²Probability for acid effect.

³Probability for technique effect.

⁴Estimated by exponential decay of ¹³C/¹²C ratio over time.

⁵Estimated by difference between fractional rates of clearance and passage.

⁶Estimated by the exponential decay in rumen Co concentration over time.

mean that ¹³C was absorbed as part of the same infused VFA molecule. Sutton et al. (2003) using continuous infusion of ¹⁴C-labeled VFA in Friesian cows eating a diet with 60% concentrate and 40% roughage based on DM found that from the total carbon entered in the acetate pool, 1.6% went to propionate and 20.6% went to the butyrate pool. From the total carbon entered in the propionate pool, 8.5% went to acetate and 1.5% went to the butyrate pool. In the case of the total carbon in the butyrate pool, 37.3% went to acetate and 13.7% went to the propionate pool supporting the hypothesis that interconversion could contribute to the similarity among rates found in the current work.

The effect of the rumen pH on the fractional absorption rates (Table 2 and Figure 2) should be considered. Dijkstra et al. (1993) did not observe a positive correlation between the carbon chain length and the absorption rate of each VFA at pH values above of 5.4. However, when the initial pH of a VFA solution introduced in an evacuated rumen of dairy cows was 4.5, the fractional HAc absorption rate was 35%/h, for HPr it was 67%/h and for HBu it was 85%/h. When the pH was 5.4, the rate for HAc was 35%/h and the rates for HPr and HBu were 54 and 53%/h, respectively. When the pH was 6.3, which was similar to the normal rumen pH obtained in this experiment (Table 1), the rate for HAc was 33%/h, whereas that for HBu was 46%/h, lower than that for HPr (51%/h). When the pH was 7.2, the rate of HBu was further reduced compared with that of HPr. In the present experiment, HPr had the highest fractional rumen wall absorption rate among the studied VFA. Rumen pH values in dairy cows are usually above 5.4 (Garrett et al., 1999), which might mean that HPr is more quickly absorbed in vivo than other VFA. Interconversion among VFA also seems to be influenced by pH. In a diet with low roughage content, lower pH was associated with greater percentage of carbon migrating from acetate and butyrate to propionate. How-

ever, the lower pH reduced the percentage of carbon migrating from butyrate and propionate to acetate (Sutton et al., 2003). Dijkstra et al. (1993) found a much higher numerical value for the fractional absorption rates than that found in this study (between 14.2 and 19.2%/h). It might be because those authors used the technique of rumen evacuation and the introduction of a VFA solution in an empty rumen. This probably increased the relation between the rumen wall absorptive surface and the volume of solution, compared with the mixture of the markers to the rumen digesta.

The fractional passage rate of the rumen fluid was around 15%/h (Table 2). The disappearance of VFA via passage corresponded to about 50% of the total clearance. This value is similar to the estimate of Peters et al. (1990), which was based on the continuous infusion of HPr in steers consuming food. A good estimate of the passage rate is essential for interpretation of the results of the techniques used. The methodology in the present work has permitted the estimate of the passage rate with high precision. The principal problem with fluid markers is the mixing especially when authors use infusion without rumen evacuation (Allen et al., 2000; Sutton et al., 2003). In the current experiment the mean of the determination coefficients obtained in all exponential cobalt decay curves was 0.98, with a range from 0.94 to 0.99, indicating that the marker solution mix and fluid collection point were not a problem. The nutritional manipulation of the absorption capacity of the rumen wall (Dirksen et al., 1984) has potential as a tool for the rumen VFA pool control; however, in cattle the manipulation of the fluid passage rate has a similar performance potential. A better understanding of the absorption of VFA in omasum and abomasum (Rupp et al., 1994) can contribute to the understanding of metabolic disturbances resulting from an excessive VFA production in the rumen (Svendsen, 1969). Methods which measured the absorptive capacity through

Figure 2. Fractional clearance rates (kc), fractional passage rates (kp), and fractional absorption rates (ka) estimated with the valerate-cobalt (HVal-Co) technique and rumen pH in Experiment 1 (■) and Experiment 2 (□): Rumen pH = $4.93 + 0.0328$ HVal-Co kc; $r^2 = 0.43$; $P < 0.001$; rumen pH = $5.18 + 0.0578$ HVal-Co kp; $r^2 = 0.45$; $P < 0.01$; rumen pH = $5.51 + 0.0302$ HVal-Co ka; $r^2 = 0.17$; $P = 0.05$.

rumen evacuation followed by the introduction of VFA solutions in the empty rumen (Sutton et al., 1963; Dijkstra et al., 1993) can not represent the actual capacity of VFA clearance from the organ as well.

No differences were detected between techniques in the estimates of the fractional clearance rate and fractional absorption rate of HVal obtained simultaneously in Experiment 2 (Table 2); thus, showing the estimate precision between techniques. The absorption values of HVal obtained in Experiment 2 were similar to the estimates for HAc, HPr, and HBu obtained in Experiment 1. As already argued, the effect of rumen pH might have contributed to this similarity. The HVal fractional clearance rate estimated by using the HVal-Co technique in the 18 infusions in Experiment 1 was 34.3%/h and the fractional absorption rate was 19.0%/h. The precision of the HVal-Co technique was high between experiments. Allen et al. (2000) obtained estimates of the fractional absorption rate of HVal varying from 16 to 25%/h using infusion of unlabeled HVal in the rumen of nonpregnant and nonlactating Holstein cows previously fed with low or highly fermentable diets. These rates were similar to the estimates found in this work.

The absorption of ME from rumen VFA was estimated to evaluate the accuracy of the clearance fractional rates (Table 3). The volume of rumen liquid was estimated by intercepts of the exponential equation describing the decline in the rumen Co concentration with time and from rumen digesta weight in the evacuation corrected for its DM content. The volume of rumen liquid estimated from the rumen evacuation was higher than the volume obtained from Co dilution at zero time ($P < 0.01$ in both experiments). This would suggest that Co did not have access to the liquid inside digesta particles. Sutton et al. (2003) could not obtain coherent values for rumen water volumes by use of 2 liquid-phase markers, but in our experiments, the values showed a similar pattern of volume changes to that measured by emptying. This may be because of better homogenization of the markers or a more adequate sampling pattern. The rumen VFA pool was calculated by multiplying the volume of rumen liquid, obtained by Co dilution methods, by the mean concentration of HAc (80.3 mM), HPr (24.3 mM), and HBu (13.7 mM) of all samples obtained at time zero. The daily flow of each VFA (mol/d) was calculated with the fractional clearance rate of each acid (Table 2), and with the fractional clearance rate of HVal estimated simultaneously by HVal-Co technique in each group of cows that had received labeled acid. These values were 35.0, 35.0, and 33.1%/h for the group of cows that had received labeled HAc, HPr, and HBu, respectively, and were assumed to be representative for the fractional clearance rate of each of these acids.

Table 3. Estimated flow of acetate (HAc), propionate (HPr), and butyrate (HBu) from rumen volume, rumen VFA concentration at time zero and fractional clearance rate of HAc, HPr, and HBu estimated by ^{13}C -VFA technique, and with the valerate-cobalt (HVal-Co) technique in Experiment 1

	Technique	
	^{13}C -VFA	HVal-Co
Acetate (mol/d)	55.3	62.1
Propionate (mol/d)	17.9	18.8
Butyrate (mol/d)	9.2	10.0
VFA/DMI ¹ (mol/kg)	4.7	5.1
Acetate ² (Mcal/d)	11.6	13.0
Propionate ² (Mcal/d)	6.6	6.9
Butyrate ² (Mcal/d)	4.8	5.2
Total VFA (Mcal/d)	23.0	25.1
ME from VFA ³ (% of requirement)	52.3	57.2
CC ⁴ from acetate (% of total)	50.4	51.7
CC from propionate (% of total)	28.6	27.4
CC from butyrate (% of total)	21.0	20.9

¹DMI = 17.7 kg/d.

²The ME flow was calculated assuming the following heat of combustion for acetate, propionate, and butyrate, respectively: 209, 367, and 524 kcal/mol.

³The ME requirement for maintenance and production was calculated considering a cow weighing 568 kg, producing 25.7 kg/d of milk with 4.11% of fat, 3.03% of protein, and 4.34% of lactose. Requirement = 43.88 Mcal of ME/d (NRC, 2001).

⁴CC = Caloric contribution of each VFA.

The daily absorption of ME derived from HAc, HPr, and HBu estimated with the ^{13}C -VFA technique was 91% of the value estimated for the HVal-Co technique (Table 3). Depending on the calculation method, the energy derived from these acids was found to be responsible for 52 to 65% of the ME requirement for both maintenance and production of the average cow. Bergman (1990), in reviewing the literature on this subject, concluded that 65 to 75% of the ME requirements of ovines derive from ruminal HAc, HPr, and HBu. This author provided evidence that the HAc corresponds to about 50% of the rumen VFA caloric flow, whereas HPr and HBu contribute about 25% each. Sutton et al. (2003), using ^{14}C -VFA technique, calculated that caloric contribution as net energy from VFA was about 55% from acetate, 29% from propionate, and 16% from butyrate in cows eating 60% of concentrate and 40% of roughage. In the current work, the caloric contribution as ME was 50 and 52% for acetate, 29 and 27% for propionate, and 21 and 21% for butyrate, estimated from ^{13}C and HVal-Co techniques, respectively.

In the present work the HAc, HPr, and HBu absorption estimate for each kilogram of ingested DM ranged from 4.7 to 5.9 mol/kg. If these 3 VFA are assumed to represent about 95% of the total VFA produced in rumen, the estimate of the total VFA production per kilogram of ingested DM ranged from 4.9 to 6.2 mol/kg. In his review, Bergman (1990) concluded that the daily

VFA production in the rumen is approximately 5 mol/kg of ingested DM. In dairy cows fed on winter diets composed of grass hay and concentrates, at 12.9 and 12.7 kg of DMI, Sutton et al. (2003) calculated VFA productions of 8.7 and 9.6 mol/kg of digestible DM. Grass fed in the current experiments had a digestibility of 80%, and taking that into account would result in 6.1 to 7.8 mol of VFA/kg of digestible DM, slightly lower than the estimates of Sutton et al. (2003). This could at least partly be explained by interchange of C between acids. It may be assumed that this interchange results in some losses of ^{13}C to the CO_2 pool notably during the conversions of acids with an even number of C atoms (HAc and HBu) to acids with an odd number (HPr and HVal). The magnitude of the fractional clearance rates determined in this work by the ^{13}C -VFA technique (and by the HVal-Co technique) seems to be biologically coherent.

The k values of each VFA estimated from HVal-Co could be an option to correct the small overestimate of VFA flow when the HVal fractional clearance rate estimated by the HVal-Co technique is used to calculate the flow of HAc, HPr, and HBu (Table 3). To estimate these rates, the regression of the fractional clearance rates was generated from the data of each ^{13}C -labeled VFA compared with the data obtained from the HVal-Co technique in each cow and each period (Figure 3 and Table 4). The quality of the regressions was proportional to the chemical similarity between the used VFA as dependent variable and the HVal. However, the regressions between the data generated with ^{13}C -valerate and the data from the HVal-Co technique in Experiment 2 ($\text{kc } ^{13}\text{C valerate} = 23.38 + 0.2984 \text{ HVal}$) had an $r^2 = 0.20$ and $P = 0.37$, showing that the chemical similarity between acids is not a unique variable which determines the quality of the regressions (Figure 4). The methodology of mathematical adjustment can be damaged by the fact that the data from HAc, which is the VFA responsible for 50% of the absorbed caloric contribution, has the lowest correlation with the data from unlabeled HVal. These regressions require validation from an independent database.

The correlation between the rumen disappearance of HVal estimated by the HVal-Co technique and mean rumen pH was evaluated by using the 18 observations from Experiment 1 and the 6 from Experiment 2 (Figure 2). Clearance, absorption, and passage rates were positively correlated with rumen pH ($\text{pH} = 4.93 + 0.0328 \text{ kc HVal}$; $r^2 = 0.43$, $P < 0.001$; $\text{pH} = 5.18 + 0.0578 \text{ kp HVal}$; $r^2 = 0.45$, $P < 0.001$; $\text{pH} = 5.51 + 0.0302 \text{ ka HVal}$; $r^2 = 0.17$, $P = 0.05$, where kc, kp, and ka represent the fractional rates of clearance, passage, and absorption, respectively). Higher rumen liquid volume was negatively associated with the HVal fractional disappear-

Figure 3. Fractional clearance rates (kc) of acetate, propionate, and butyrate. Correlation between estimates obtained with ^{13}C -labeled VFA and the valerate-cobalt (HVal-Co) technique: ^{13}C -acetate $\text{kc} = 5.19 + 0.7427 \text{ HVal-Co kc}$; $r^2 = 0.51$; $P = 0.07$; ^{13}C -propionate $\text{kc} = 19.17 + 0.4073 \text{ HVal-Co kc}$; $r^2 = 0.56$; $P = 0.08$; ^{13}C -butyrate $\text{kc} = 7.21 + 0.7000 \text{ HVal-Co kc}$; $r^2 = 0.99$; $P < 0.01$ (Experiment 1). Dashed line shows the equality line.

Table 4. Regression equations¹ generated from the data of each ^{13}C -labeled VFA² against the data obtained from the HVal-Co³ technique in each cow and each period

kc ⁴	A	b	R ²	P
$^{13}\text{C-HAc}$	5.19	+0.7427	0.51	0.07
$^{13}\text{C-HPr}$	19.2	+0.4073	0.56	0.08
$^{13}\text{C-HBu}$	7.21	+0.7000	0.99	<0.01

¹Regression parameters: $y = A + bx$ of the kc ^{13}C (y) on the kc HVal-Co (x) technique.

² $^{13}\text{C-HAc}$, $^{13}\text{C-HPr}$, $^{13}\text{C-HBu}$ = labeled acetate, propionate, and butyrate, respectively.

³HVal-Co = Valerate-cobalt.

⁴kc = Fractional clearance rate.

ance rate (Figure 5; clearance = $58.89 - 0.2684$ rumen fluid volume; $r^2 = 0.37$; $P < 0.01$; passage = $28.25 - 0.1431$ rumen fluid volume; $r^2 = 0.31$ $P < 0.01$; absorption = $29.07 - 0.1184$ rumen fluid volume; $r^2 = 0.16$; $P = 0.05$). Cows with higher rumen liquid volume had a lower pH (Figure 6) ($\text{pH} = 7.73 - 0.0184$ rumen fluid volume; $r^2 = 0.70$; $P < 0.001$). The fact that a higher fractional passage rate was associated with the higher rumen pH (Figure 2) seems to imply that the high rumen pH was a consequence of the higher VFA fractional clearance rate. Based on these data, it can be speculated whether lactating cows, which have higher daily DM

Figure 4. Correlation between the fractional clearance rate (kc) of valerate estimated with the ^{13}C -labeled valerate (HVal) technique and the estimate obtained with the valerate-cobalt (HVal-Co) technique: $^{13}\text{C-HVal kc} = 23.38 + 0.2984 \text{ HVal-Co kc}$; $r^2 = 0.20$; $P = 0.37$ (Experiment 2). Dashed line shows the equality line.

Figure 5. Fractional clearance rates (kc), fractional passage rates (kp), and fractional absorption rates (ka) estimated with the HVal-Co technique and the mathematically estimated rumen volume in Experiment 1 (■) and Experiment 2 (□): HVal-Co kc = $58.89 - 0.2684$ rumen volume; $r^2 = 0.37$; $P < 0.01$; HVal-Co kp = $28.25 - 0.1431$ rumen volume; $r^2 = 0.31$ $P < 0.01$; HVal-Co ka = $29.07 - 0.1184$ rumen volume; $r^2 = 0.16$; $P = 0.05$.

Figure 6. Mean rumen pH and the mathematically estimated rumen volume in Experiment 1 (■) and Experiment 2 (□): Rumen pH = $7.73 - 0.0184$ rumen volume; $r^2 = 0.70$; $P < 0.01$.

consumption than nonlactating cows and thus have a higher rumen volume, could have a lower fractional VFA absorption rate and greater propensity for a low rumen pH. However, experiments showing measurements of the fractional VFA clearance rates in nonlactating cows compared with lactating ones are unknown.

CONCLUSIONS

The VFA clearance and fractional absorption rates obtained by the stable isotope technique gave similar results to those obtained by the HVal-Co technique, indicating that both techniques present reliable results for VFA rumen clearance determinations and ME absorption calculations.

Linear equations based on estimates of clearance of unlabeled HVal obtained by the HVal-Co technique can be useful in the prediction of the fractional clearance rates of HAc, HPr, and HBu. However, the regressions were better for HBu than for HAc and HPr.

The quantitative importance of the fractional passage rate is similar to the fractional absorption rate in the VFA clearance of dairy cows.

ACKNOWLEDGMENTS

The authors are grateful to the CAPES foundation (Brazil) for financial support. We also would like to

thank Jan Dijkstra for his useful suggestions. The authors thank the staff of the Animal Nutrition group especially Dick Bongers, Toos Lammers, Truus Post, Bart Tas, Harm Smit, and Hassan Taweel for their direct contributions to the experiments.

REFERENCES

- Allen, M. S., L. E. Armentano, M. N. Pereira, Y. Ying, and J. Xu. 2000. Method to measure fractional rate of volatile fatty acid absorption from the rumen. Page 26 in Proc. 25th Conf. Rumen Function, Chicago, IL.
- Barker, I. K., A. A. Van Dreumel, and N. Palmer. 1995. The alimentary system. Page 1 in Pathology of domestic animals. 4th ed. Vol 2. K. V. F. Jubb, P. C. Kennedy, N. Palmer, ed. Academic Press, San Diego, CA.
- Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70:567–590.
- Boutton, T. W. 1991. Stable carbon isotope ratios of natural material. I. Sample preparation and mass spectrometric analysis. Pages 155–171 in Carbon Isotope Techniques. D. C. Coleman and B. Fry, ed. Academic Press, New York, NY.
- Chen, X., S. Tamminga, H. Boer, P. Chilibroste, and T. Lammers. 1997. Het meten van vluchtige vetzuurproducties m.b.v. stabiele isotopen in de pens van koeien. Pages 76–77 in Proceedings Studiedag Nederlandstalige Voedingsonderzoekers, Wageningen, Wageningen Acad. Publ., Wageningen, The Netherlands.
- Crichlow, E. C., and R. K. Chaplin. 1985. Ruminant lactic acidosis: Relationship of forestomach motility to nondissociated volatile fatty acids levels. *Am. J. Vet. Res.* 46:1908–1911.
- Dijkstra, J., H. Boer, J. V. Bruchem, M. Bruining, and S. Tamminga. 1993. Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *Br. J. Nutr.* 69:385–396.
- Dirksen, G., H. G. Liebich, G. Brosi, H. Hagemeister, and E. Mayer. 1984. Morphologie der Pansenschleimhaut und Fettsäureresorption beim Rind – bedeutende Faktoren für Gesundheit und Leistung. *Zentralbl. Veterinärmed.* 31:414–430.
- Elliot, J. P., J. K. Drackley, G. C. Fahey, and R. D. Shanks. 1995. Utilization of supplemental fat by dairy cows fed diets varying in content of nonstructural carbohydrates. *J. Dairy Sci.* 78:1512–1525.
- Gabel, G. 1995. Transport of short-chain fatty acids in the ruminant forestomach. Pages 133–147 in Physiological and clinical aspects of short-chain fatty acids. J. H. Cummings, L. Rombeau, and T. Sakata, ed. Cambridge University, Cambridge, UK.
- Garrett, E. F., M. N. Pereira, K. V. Nordlund, L. E. Armentano, W. J. Goodger, and G. R. Oetzel. 1999. Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. *J. Dairy Sci.* 82:1170–1178.
- Grant, R. J., and D. R. Mertens. 1992. Influence of buffer pH and raw cornstarch addition on in vitro fiber digestion kinetics. *J. Dairy Sci.* 75:2762–2768.
- Gray, F. V., A. F. Pilgrim, H. J. E. Rodda, and R. A. Weller. 1952. The nature and origin of the volatile fatty acids in the rumen of the sheep. *J. Exp. Biol.* 29:57–68.
- Hoover, W. H. 1986. Chemical factors involved in ruminal fiber digestion. *J. Dairy Sci.* 69:2755–2766.
- Huntington, G. B., P. J. Reynolds, and H. F. Tyrrell. 1983. Net absorption and ruminal concentrations of metabolites in nonpregnant dry Holstein cows before and after intraruminal acetic acid infusion. *J. Dairy Sci.* 66:1901–1908.
- ISO Analytical. 2002. Beginners guide for stable isotope analysis. Available: <http://www.iso-analytical.com/page21.html> ISO Analytical, Ltd., Sandbach, Cheshire, UK.
- Jensen, R., H. M. Deane, L. J. Cooper, V. A. Miller, and W. R. Graham. 1954. The rumenitis-liver abscess complex in beef cattle. *Am. J. Vet. Res.* 15:202–204.
- Kristensen, N. B. 2001. Rumen microbial sequestration of [2-¹³C]acetate in cattle. *J. Anim. Sci.* 79:2491–2498.
- Nozière, P., C. Martin, D. Rémond, N. B. Kristensen, R. Bernard, M. Doreau. 2000. Effect of composition of ruminally infused short-chain fatty acids on net fluxes of nutrients across portal-drained viscera in underfed ewes. *Br. J. Nutr.* 83:521–531.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. National Academy of Science, Washington, DC.
- Pereira, M. N., and L. E. Armentano. 2000. Partial replacement of forage with nonforage fiber sources in lactating cow diets. II. Digestion and rumen function. *J. Dairy Sci.* 83:2876–2887.
- Peters, J. P., R. Y. W. Shen, and S. T. Chester. 1990. Propionic acid disappearance from the foregut and small intestine of the beef steer. *J. Anim. Sci.* 68:3905–3913.
- Robinson, P. H., S. Tamminga, and A. M. Van Vuuren. 1987. Influence of declining level of feed intake and varying the proportion of starch in the concentrate on rumen ingesta quality, composition and kinetics of ingesta turnover in dairy cows. *Livest. Prod. Sci.* 16:37–45.
- Rupp, G. P., K. K. Kreikemeier, L. J. Perino, and G. S. Ross. 1994. Measurement of volatile fatty acid disappearance and fluid flux across the abomasum of cattle, using an improved omasal cannulation technique. *Am. J. Vet. Res.* 55:522–529.
- SAS Institute. 1999. SAS/STAT Users Guide. Version 6.12. SAS Institute Inc., Cary, NC.
- Sutton, J. D., M. S. Dhanoa, S. V. Morant, J. France, D. J. Napper, and E. Schuller. 2003. Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. *J. Dairy Sci.* 86:3620–3633.
- Sutton, J. D., A. D. McGilliard, and N. L. Jacobson. 1963. Functional development of rumen mucosa. I. Absorptive ability. *J. Dairy Sci.* 46:426–436.
- Svendsen, P. 1969. Etiology and pathogenesis of abomasal displacement in cattle. *Nord. Vet. Med.* 21(Suppl.1):1.
- Tóthi, R. 2003. Processed grains as a supplement to lactating dairy cows. PhD Thesis, Wageningen Univ., Wageningen, The Netherlands.
- Uden, P., P. E. Colucci, and P. J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. *J. Sci. Food Agric.* 31:625–632.
- Yeh, H. W., and W. M. Wang. 2001. Factors affecting the isotopic composition of organic matter. (1) Carbon isotopic composition of terrestrial plant materials. *Proc. Natl. Sci. Counc. Repub. China B* 25:137–147.