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Comparison of non-tracer and tracer methods for determination of volatile fatty acid production rate in the rumen of sheep fed on two levels of intake

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The aim of the present work was to estimate volatile fatty acid (VFA) production rate in the rumen of sheep fed two levels of intake using both a tracer (TM; by isotope dilution) and a non-tracer method (NTM; by supplementary infusion) in steady-state conditions. Six wethers received a diet containing 700 g lucerne hay and 300 g ground maize/kg in eight equal meals at 3 h intervals per d. The diet (9·8 MJ metabolizable energy (ME)/kg DM) was offered at 90 % *ad libitum* consumption (high intake, HI) or 45 % *ad libitum* consumption (low intake, LI) in a 2 × 2 crossover design. Each sheep received five intrarumen VFA solutions infused continuously for 24 h at rates of 250 ml and 165 ml/h for the HI and LI respectively. The first infusion, considered as a control treatment (Con), consisted of a solution of [1-^{13}C]propionate (7 mmol/d). The four other solutions were isoenergetic (1·9 MJ ME/kg DM intake) mixtures of unlabelled propionate (C_3) and butyrate (C_4) at different levels: 0·90 mol C_4/kg DM intake; 0·60 mol C_4 + 0·45 mol C_3/kg DM intake; 0·30 mol C_4 + 0·90 mol C_3/kg DM intake; 1·35 mol C_3/kg DM intake. The VFA infusions did not affect rumen fermentation of the basal diet (pH, osmotic pressure, protozoa numbers), and comparable DM digestibility of the diet among the different treatments was observed. Both estimation methods demonstrated a similar increase (1·7-fold) in the rumen VFA production rate of sheep fed at intakes varying between 0·9 to 1·7 times maintenance. Irrespective of the intake level, the rumen production rate of individual VFA was on average 1·5-fold higher when estimated by the TM compared with the NTM. Rumen VFA production rates estimated by the NTM and TM represented 80 % and 120 % ME intake respectively. The difference between NTM and TM estimates seems likely to be caused mainly by overestimation of the VFA production rates by the TM.

Rumen: Volatile fatty acids: Production rate: Intake level

Volatile fatty acids (VFA) are the main energy source for ruminants, contributing 50–80 % of the total energy supply (Thomas & Clapperton, 1972; Sutton, 1985). The major part (75 %) of the rumen VFA disappears across the reticulorumen wall with a variable absorption rate, especially when concentrate diets are fed. This means that the rumen VFA concentration is not directly proportional to their production rate (Esdale *et al.* 1968; Sharp *et al.* 1982). Assessment of the quantitative contribution of VFA to ruminant nutrition requires measurements of the VFA production rate in the rumen. In general, two groups of techniques are employed for *in vivo* determination of rumen VFA production rates: tracer methods (TM) and non-tracer methods (NTM). TM usually use an isotope-dilution technique of a solution of VFA labelled with radioactive isotopes (^{14}C,^{3}H) and continuously infused into the rumen at a constant rate (Leng & Leonard, 1965; Leng & Brett, 1966; Bruce *et al.* 1987). The radioactivity of these compounds has been considered to be a major disadvantage of *in vivo* experiments and Breves *et al.* (1987) proposed the use of a stable isotope (^{13}C). A variety of NTM is used to quantify VFA production rate in the rumen, including CH_4 production, portal–arterial difference, and perturbation of the steady state. CH_4 production is an index of rumen

Abbreviations: HI, high intake level; LI, low intake level; ME, metabolizable energy; NTM, non-tracer method; OM, organic matter; TM, tracer method; VFA, volatile fatty acid.

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fermentation used to obtain indirect estimates of VFA production. The portal–arterial difference in VFA concentration precludes accurate estimation of rumen VFA production, because metabolism of VFA in the rumen wall is not considered. The perturbation of steady state method relies on changes in VFA rumen concentration when one or a mixture of acids is infused at a constant rate in steady-state conditions (Bath *et al.* 1962; France & Siddons, 1993). More recently, Huhtanen & Jaakkola (1995) suggested a technique with increasing levels (at least three) of VFA infusion into the rumen. Rumen VFA production rate can then be estimated using a regression approach. This estimation is based on the assumptions that there is an equilibrium between rumen concentration and rate of absorption, and that the infusions do not alter the rumen fermentation pattern and digestion of the diet.

Since no direct simultaneous comparison of different techniques appears to have been published, comparison of the results obtained using these different methods is hampered by differences in the experimental conditions (animals, diets, level of intake, type of isotope). Moreover, most of the studies on the estimation of VFA production rates aimed to improve the precision of the technique, while the main factors affecting the rumen production rate (intake level, nature of the diet) have received little attention (Leng & Brett, 1966; Esdale *et al.* 1968; Bauman *et al.* 1971). Thus, the aim of the present study was to estimate VFA production rates in the rumen of sheep fed two levels of intake, and to define more clearly the utilization limits of the TM and NTM for *in vivo* estimation of rumen VFA production rates.

Materials and methods

Animals, feeding and experimental procedures

Texel wethers (*n* 6, 2 years old) with an average body weight 73·2 (SD 2·8) kg at the start of the experiment and 73·3 (SD 5·5) kg body weight at the end of the experiment were used. The animals were fitted with a rumen cannula (i.d. 60 mm) made of polyamide and polyvinyl chloride (Synthesia, Nogent sur Marne, France). Surgery was performed under general anaesthesia (Halothane; ICI Pharma-veterinaire, Paris, France). The sheep were allowed 4 weeks to recover after surgery. During the experiment, the sheep received a diet containing 700 g chopped lucerne hay (914 g organic matter (OM), 144 g crude protein and 571 g neutral detergent fibre/kg DM)/kg and 300 g ground maize (988 g OM, 91 g crude protein and 131 g neutral detergent fibre/kg DM)/kg in eight equal meals at 3 h intervals per d with an automatic feeder throughout the experiment. The diet supplied 9·80 MJ metabolizable energy (ME)/kg DM intake and was offered at 1550 (SD 75) g DM, corresponding to 90 % *ad libitum* consumption (high intake, HI), or at 810 (SD 5) g DM, corresponding to 45 % *ad libitum* consumption (low intake, LI). The animals were allocated to two groups (three sheep in each group) and to the two levels of DM intake according to a crossover design, whereby both of the two levels of DM intake were fed to all six sheep.

The animals were housed individually in metabolism cages in a room with continuous lighting and air conditioning, and had free access to lick mineralized salt blocks. The sheep did not have free access to water. Instead, water was continuously infused into the rumen, using a six-channel peristaltic pump (Minipuls; Gilson, Villers le Bel, France), in amounts corresponding to the average daily *ad libitum* consumption determined during the adaptation period using a water meter. Continuous intrarumen infusion of water and distribution of feed at short intervals were chosen as optimal steady state conditions in the rumen to avoid random variations in microbial fermentation, liquid pool size and consequently VFA concentrations. The experiment consisted of two periods. Each period lasted 6 weeks, comprising a 3-week adaptation period followed by a 3-week measurement period.

Volatile fatty acid infusions

Each sheep received five VFA solutions infused continuously into the rumen using a peristaltic pump. Each infusion lasted 24 h and the total volume infused was the same as the average water intake during the adaptation period (i.e. 6 litres/d and 4 litres/d for the HI and LI respectively). The first infusion consisted of a solution of [1-^{13}C]propionate (0·525 g/d, i.e. 0·007 mol [1-^{13}C]propionate/d, 99·9 % enriched; Leman, St Quentin en Yvelines, France). Since exchange of propionic acid C with other short-chain fatty acids is quantitatively insignificant (Peters *et al.* 1990), [^{13}C]-propionate was chosen as the reference to predict the *in vivo* production rate of all VFA. Considering the negligible amounts of [1-^{13}C]propionate infused, the first infusion was also considered as a control treatment (Con). The four other solutions infused were made of four mixtures of non-labelled propionate (C_3) and butyrate (C_4) at different levels: 0·90 mol C_4/kg DM intake (B); 0·60 mol C_4 + 0·45 mol C_3/kg DM intake (Bp); 0·30 mol C_4 + 0·90 mol C_3/kg DM intake (bP); 1·35 mol C_3/kg DM intake (P). These solutions were chosen to be isoenergetic (1·9 MJ ME/kg DM intake), to avoid appetite depression, and to ensure sufficient increment in their rumen concentrations to be detected by GC. The pH of all VFA solutions was adjusted to pH 6·0 with 10 M-NaOH. This pH was chosen to be close to the rumen pH measured during the adaptation period to the diet. The effective amounts and composition of the VFA solutions infused are given in Table 1, and the design of infusions was as follows: Con, from 16.00 hours on day 4 to 16.00 hours on day 5 of week 1; B, from 16.00 hours on day 1 to 16.00 hours on day 2 of week 2; Bp, from 16.00 hours on day 4 to 16.00 hours on day 5 of week 2; bP, from 16.00 hours on day 1 to 16.00 hours on day 2 of week 3; P, from 16.00 hours on day 4 to 16.00 hours on day 5 of week 3.

Sampling procedures and measurements

Rumen liquid samples (100 ml) were taken from the dorsal, ventral and cranial sacs of the rumen via the cannula using a suction pump and a rigid plastic tube (length 400 mm; i.d. 15 mm), and then filtered with a 250 μm nylon filter.

Just before the onset of VFA infusions, samples of rumen liquid were withdrawn from each animal and used as blanks for ^{13}C and Cr-EDTA analyses.

Table 1. Characteristics of infused volatile fatty acid solutions

	High intake level					Low intake level				
Infusion…	Con	B	Bp	bP	P	Con	B	Bp	bP	P
Water (litres/d)	5·90	6·05	6·06	6·36	6·64	4·01	4·13	4·21	4·22	4·47
[1-^{13}C]propionate (mol/d)	0·007	–	–	–	–	0·007	–	–	–	–
Propionate (mol/d)	–	–	0·64	1·30	2·17	–	–	0·32	0·66	1·40
Butyrate (mol/d)	–	1·18	0·90	0·46	–	–	0·66	0·44	0·24	–
Metabolizable energy (MJ/d)	–	2·72	3·05	3·05	3·31	–	1·53	1·50	1·56	2·15

Con, 0·007 mol/d [1-^{13}C]propionate; B, 0·90 mol butyrate/kg DM intake; Bp, 0·60 mol butyrate + 0·45 mol propionate/kg DM intake; bP, 0·30 mol butyrate + 0·90 mol propionate/kg DM intake; P, 1·35 mol propionate/kg DM intake.

The pH of the rumen liquid was measured immediately on six samples taken 16·5, 18·0, 19·5, 21·0, 22·5 and 24·0 h after the start of each infusion using a digital pH-meter (CG840, Ag–AgCl electrode; Schott Geräte, Hofheim, Germany). These sampling times were chosen in order to have three samples at feeding times and the three others between two meals. A 10 ml portion (2 × 5 ml) from each sample of rumen fluid was mixed with 0·1 volume orthophosphoric acid (10 ml/l) and frozen (−20°C) before VFA analysis by GC using 4-methylvaleric acid as internal standard (Jouany, 1982). For the control treatment (Con), two more 5 ml portions were preserved as described earlier and used to determine the ^{13}C enrichment of rumen propionate using 2-chloroethylesters of propionate by GC–isotope ratio MS (Kristensen, 2000). A portion of 8 ml was also frozen (−20°C) for osmotic pressure measurements made with a vapour pressure osmometer (KNAUER D1000, Berlin, Germany) on particle-free rumen fluid obtained by centrifugation at 20 000 ***g*** for 10 min at 4°C. Protozoa were counted in samples collected 22·5 h after the start of infusions. For this, 1 ml rumen fluid was preserved at 4°C with 3 ml preservative solution (glycerol–formaldehyde–distilled water (50:2:48, by vol.)). Protozoa were counted using a Dolfuss cell (Elvetec Services, Clermont-Ferrand, France) according to the procedure described by Jouany & Senaud (1982). The volume and turnover rate of the rumen liquid phase was determined from a pulse dose of Cr-EDTA solution (140 mg Cr in 50 ml water), prepared according to the procedure of Binnerts *et al.* (1968), and injected into the rumen of each sheep via the cannula just before the beginning of each VFA infusion. Rumen liquid samples were taken and filtered as described previously, just before infusion and 3·0, 4·5, 6·0, 7·5, 13·5, 16·5, 19·5 and 22·5 h after the beginning of the infusion, and 20 ml liquid were frozen (−20°C) until analysed. The Cr content of samples was determined by atomic absorption spectrometer (Perkin-Elmer 2380; Perkin-Elmer Bois d'Arcy, France) after centrifugation of samples at 5000 ***g*** for 15 min at 4°C.

Feed intake and refusals were measured and recorded daily during the experimental period to calculate DM intake. Total tract digestibility was determined from daily total collection of faeces during all the 3-week infusion periods and each week corresponded to the following combinations: Con (control treatment), B and Bp (the highest butyrate:propionate ratio infused), bP and P (the highest propionate:butyrate ratio infused). Faeces were weighed and mixed before sampling a 50 % aliquot for each sheep. After DM determination (80°C for 48 h), faecal samples were pooled for each week and each sheep. The chemical composition of experimental feeds and faecal samples was determined according to Association of Analytical Chemists procedures (Association of Analytical Chemists, 1990). Gross energy of alfalfa hay and maize was obtained from Institut National de la Recherche Agronomique feed tables (Institut National de la Recherche Agronomique, 1989) and the ME of the diet was calculated from the equations of Andrieu & Demarquilly (1987).

Calculations and statistical analyses

The volume and the turnover rate of the rumen liquid phase were calculated from the exponential decrease of Cr concentrations with time. After semi-logarithmic line-fitting, the slope represented the fluid dilution rate (/h), and the volume of liquid phase in the rumen was calculated from the intercept at t 0.

For the TM, the rumen production rate of propionate (mol/d) was calculated using steady-state tracer dilution kinetics of the [1-^{13}C]propionate. The C flux through the rumen propionate pool is measured and then divided by three to obtain the rumen propionate flux according to the following equation:

$$(\text{AF infusate} - \text{AF background}) \times \text{IR}/((\text{AF rumen} - \text{AF background}) \times 3),$$

where IR is the infusion rate of [1-^{13}C]propionate solution (mol/d), and AF corresponds to the atomic fraction of $^{13}\text{C} = {}^{13}\text{C}/({}^{12}\text{C} + {}^{13}\text{C})$ for the infused [1-^{13}C]propionate solution (AF infusate), for the rumen samples before (AF background) and during infusion at equilibrium (AF rumen).

For the NTM, the regression approach of Huhtanen & Jaakkola (1995) adapted from Bath *et al.* (1962) was used to estimate the propionate and butyrate production rates by dividing the intercept a by the slope b of the regression:

$$y = \text{a} + bx$$

where y is the concentration (mmol/l) or molar proportion (%) of propionate or butyrate present in the rumen liquid, and x is the amount of propionate or butyrate infused (mol/d). The slope of the regression equations obtained between concentration and proportion of propionate or butyrate and their infusion rate were tested by analysis of covariance.

For both methods, production rates of other individual

Table 2. Effect of volatile fatty acid infusions† on number and composition of the protozoal population‡
(Mean values with their standard errors for six sheep)

	High intake level					Low intake level						Statistical significance of effects		
Infusion†…	Con	B	Bp	bP	P	Con	B	Bp	bP	P	SE	Infusion (I)	Intake level (L)	Interaction (I × L)
Protozoal counts ($\times 10^3$/ml)	410	394	367	397	366	253	225	195	244	200	32	NS	***	NS
Protozoal genera (%)														
Entodinium	76·4	71·6	75·7	67·4	68·0	81·0	82·0	78·6	74·1	79·7	4·1	NS	**	NS
Epidinium	18·8	21·9	17·0	21·2	24·5	10·5	10·7	13·6	18·9	13·4	3·5	NS	**	NS
Eudiplodinium	1·6	2·0	2·4	3·0	2·2	2·0	1·4	2·2	1·8	1·5	0·4	NS	NS	NS
Diplodinium	2·3	3·4	3·8	7·6	4·0	5·1	3·8	4·2	3·5	4·7	1·1	NS	NS	NS
Isotricha	0·6	0·6	0·8	0·4	1·0	0·7	0·5	0·6	0·8	0·4	0·2	NS	NS	NS
Dasytricha	0·3	0·6	0·4	0·4	0·3	0·7	1·5	0·8	0·8	0·4	0·3	NS	*	NS

* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† Con, 0·007 mol [1-^{13}C]propionate/d; B, 0·90 mol butyrate/kg DM intake; Bp, 0·60 mol butyrate + 0·45 mol propionate/kg DM intake; bP, 0·30 mol butyrate + 0·90 mol propionate/kg DM intake; P, 1·35 mol propionate/kg DM intake (for details, see Table 1).
‡ For details of diets and procedures, see p. 332.

VFA (VFA_i) were respectively obtained by multiplying the propionate (or butyrate) production rate by the VFA_i: propionate (or butyrate) concentration ratio.

Data were analysed by ANOVA with the following factors: period, animal, infusion, intake level and the interaction infusion×intake level. For each animal, values for pH, osmotic pressure, individual and total VFA concentrations, ^{13}C–enrichment of rumen propionate within a treatment were the mean values for the different sampling times (n 6). The effect of the nature of VFA infused for the NTM (C_3 *v.* C_4) and the effect of the method (NTM *v.* TM) to estimate the rumen VFA production rate were tested by ANOVA with period, sheep, intake level, method, method nested within VFA infusion nature, and the interaction method×intake level. The relation between the production rate of propionate estimated by the NTM and TM was tested by a covariance analysis including TM, intake level and the interaction TM×intake level as cofactors. Data were statistically analysed using the GLM procedure of SAS (SAS/STAT® User's Guide, Release 6.03, 1988; Statistical Analysis Systems Institute Inc., Cary, NC, USA). Differences were tested using the Duncan's test and declared significant at $P < 0·05$.

Results

Rumen variables and digestibility

On average, for the 3 weeks of measurements, the DM digestibilities of the diet were 67·2 (SD 0·5) and 69·5 (SD 1·8) % respectively, for animals fed the HI and LI ($P > 0·05$). Comparable DM digestibilities of the diet were observed with the control treatment (Con) for the HI (67·6 %) and LI (67·9 %). No effect of the infused mixtures of unlabelled propionate and butyrate was observed on the digestibilities of the diet for the two intake levels ($P > 0·05$), although the VFA infusions richest in butyrate (B and Bp) tended to increase DM digestibility (71·4 %) compared with the other infusions for animals fed at LI.

The protozoal number in the rumen was higher ($P < 0·001$; Table 2) for animals fed at HI than those fed at LI (387×10^3/ml *v.* 223×10^3/ml on average respectively), and the proportion of *Entodinium* (mean value 72 *v.* 79 %, $P < 0·01$), *Epidinium* (mean value 20 *v.* 13 %, $P < 0·01$) and *Dasytricha* (mean value 0·4 *v.* 0·8 %, $P < 0·05$) differed between HI and LI. Irrespective of the intake level, the number and the composition of the protozoal population was unaffected by the different VFA infusions ($P > 0·05$; Table 2).

The rumen pH values were significantly ($P < 0·001$) lower for HI than for the LI (mean value 6·44 *v.* 6·58, Table 3). Infusions of VFA increased the rumen pH ($P < 0·001$) in the same way for the two levels of intake (infusion×intake level interaction $P > 0·05$), i.e. an increase of 0·25 pH units for HI and 0·22 pH units for LI. The osmotic presure was higher ($P < 0·001$) for HI than for LI (mean value 302 *v.* 244 mosmol/l respectively) and the effect observed for VFA infusions on the rumen osmotic pressure was different for the two levels of intake (infusion×intake level interaction $P < 0·001$, Table 3). For HI, rumen osmotic pressure increased by 92 mosmol/l between treatments Con and Bp

Table 3. Effect of volatile fatty acids infusions† on rumen physico-chemical characteristics and volatile fatty acid concentrations‡
(Mean values with their standard errors for six sheep)

	High intake level					Low intake level						Statistical significance of effects‡		
Infusion†...	Con	B	Bp	bP	P	Con	B	Bp	bP	P	SE	Infusion (I)	Intake level (L)	Interaction (I × L)
PH	6·28	6·47	6·41	6·53	6·52	6·44	6·58	6·63	6·66	6·59	0·05	***	***	NS
Osmotic pressure (mosmol/l)	248	303	340	317	301	216	246	253	253	251	5	***	***	***
Liquid volume (litres)	6·0	6·4	6·6	6·5	7·1	6·2	6·1	5·7	5·6	6·0	0·2	NS	***	*
Liquid dilution rate (/h)	0·129	0·124	0·113	0·115	0·102	0·065	0·067	0·074	0·075	0·068	0·005	*	***	*
Volatile fatty acids (mmol/l)														
Total	100·9	119·0	123·2	116·9	135·6	73·0	90·0	90·8	89·6	101·4	4·5	***	***	NS
Acetate	62·8	63·6	64·3	60·1	66·9	47·3	50·9	50·1	48·3	51·0	2·5	NS	***	NS
Propionate	20·6	19·4	28·3	32·9	47·2	13·0	14·6	20·8	24·4	35·8	1·7	***	***	NS
Butyrate	13·9	31·3	25·6	19·4	17·5	9·7	20·6	15·9	13·1	10·4	1·0	***	***	NS
Isobutyrate	1·02	1·26	1·45	1·35	1·55	0·95	1·15	1·24	1·21	1·34	0·05	***	***	NS
Valerate	1·29	1·39	1·59	1·45	1·52	0·82	0·94	0·97	0·98	1·16	0·06	***	***	NS
Isovalerate	0·96	1·33	1·46	1·38	1·54	1·00	1·36	1·38	1·31	1·42	0·06	***	NS	NS
Caproate	0·31	0·67	0·48	0·32	0·32	0·27	0·40	0·39	0·28	0·26	0·05	***	***	NS

* $P < 0·05$, *** $P < 0·001$.
† Con, 0·007 mol/d [1-^{13}C]propionate/d; B, 0·90 mol butyrate/kg DM intake; Bp, 0·60 mol butyrate + 0·45 mol propionate/kg DM intake; bP, 0·30 mol butyrate + 0·90 mol propionate/kg DM intake; P, 1·35 mol propionate/kg DM intake (for details, see Table 1).
‡ For details of diets and procedures, see p. 332.

(248 to 340 mosmol/l) and then decreased to 301 mosmol/l. For LI, the osmotic pressure increased by 37 mosmol/l between infusions Con and Bp (216 to 253 mosmol/l) and then stayed stable with the infusions highest in propionate (bP, P). The mean volumes of the rumen liquid phase were 6·5 litres and 5·9 litres for the HI and LI respectively ($P < 0·001$) and were not affected by treatments ($P > 0·05$, Table 3), although the rumen liquid volume tended to increase with the highest infusion of propionate (infusion P) at HI. Liquid dilution rate was higher ($P < 0·001$) for HI (mean value 0·117/h) than for LI (mean value 0·070/h) and decreased as propionate increased in VFA infusions ($P < 0·05$) for animals fed at HI. No effect of VFA infusion was observed on the rumen fluid dilution rate for LI (Table 3).

The rumen concentrations of individual VFA for the different treatments are presented in Table 3. The rumen concentrations of the total and individual VFA (except for isovalerate) were higher for animals receiving the basal diet (Con) at HI compared with LI. Conversely, the molar proportions of the individual VFA characterizing the basal diet were comparable between the two levels of intake (mean value 63·5 % for acetate, 19·1 % for propionate, and 13·6 % for butyrate) except for the two isoacids for which the molar proportions were lower when animals were fed at HI compared with LI (results not shown). Compared with the control treatment (Con), the rumen concentration of all VFA (except acetate) was changed ($P < 0·001$) by infusion of unlabelled acids in the same way for the two intake levels (infusion×intake level interaction $P > 0·05$). Considering only the four unlabelled VFA infusions, the response to gradual replacement of butyrate with propionate in VFA infusions was a decrease in rumen butyrate concentrations ($P < 0·001$) and an increase in rumen propionate concentrations ($P < 0·001$), whereas the rumen concentrations of acetate were not changed ($P > 0·05$). For the rumen concentration of total VFA, we observed similar concentrations for treatments B, Bp, and bP, but an increase with the highest infusion level of propionate (P), and thus for the two intake levels.

The regression equations calculated for the amount of unlabelled acids infused (mol/d) and their rumen concentration (mmol/l) or molar proportion (%) are presented in Table 4. Irrespective of the intake level, the slopes of the regression equations obtained from the concentration and molar proportion were comparable for the butyrate ($P > 0·05$), but lower for the propionate ($P < 0·01$ and $P < 0·05$ for the HI and LI respectively) when estimated from proportions instead of from concentrations. This latter observation suggested an increase in the absorption rate of propionate with the highest infusion rate of propionate (treatment P). The quantity of propionate infused with infusion P was considerable and corresponded to 1·4 mol C_3/kg DM intake or 22 % ME intake for HI and 1·7 mol C_3/kg DM intake or 27 % ME intake for LI. So, the regression equations were recalculated without results from the infusion P and the comparable slope of the new regression equations (Table 4) confirmed our hypothesis. According to these results, modifications in the rumen conditions may occur for a quantity of propionate infused exceeding 1 mol/kg DM intake or 20 % ME intake. Moreover, variations in VFA concentrations may result

Table 4. Variables of the regression equations between the rumen concentration or molar proportion of propionate or butyrate and their infusion rate*

(Mean values with their standard errors for six sheep)

	High intake level						Low intake level					
	Infusion rate (mol/d)	Intercept		Slope			Infusion rate (mol/d)	Intercept		Slope		
		Mean	SE	Mean	SE	*R*		Mean	SE	Mean	SE	*R*
From infusions Con, B, Bp, bP, P†												
Propionate (mM)	0–2·17	19·5	1·3	12·3	1·1	0·90	0–1·40	14·3	0·5	15·5	0·8	0·97
Propionate (mol/100 mol)	0–2·17	17·7	0·7	7·7	0·5	0·94	0–1·40	17·2	0·5	13·7	0·7	0·96
Butyrate (mM)	0–1·18	15·2	0·9	12·6	1·3	0·87	0–0·66	9·8	0·5	14·7	1·3	0·90
Butyrate (mol/100 mol)	0–1·18	12·8	0·7	9·7	1·0	0·88	0–0·66	11·5	0·6	15·3	1·5	0·89
From infusions Con, B, Bp, bP†												
Propionate (mM)	0–1·30	20·3	0·6	10·2	0·9	0·92	0–0·66	14·2	0·6	16·4	1·6	0·91
Propionate (mol/100 mol)	0–1·30	17·6	0·9	8·2	0·7	0·90	0–0·66	16·7	0·6	16·5	1·5	0·92

* For details of diets and procedures, see p. 332.
† Con, 0·007 mol/d [1-^{13}C]propionate; B, 0·90 mol butyrate/kg DM intake; Bp, 0·60 mol butyrate + 0·45 mol propionate/kg DM intake; bP, 0·30 mol butyrate + 0·90 mol propionate/kg DM intake; P, 1·35 mol propionate/kg DM intake (for details, see Table 1).

from sampling or differences in the rumen fluid volume, as observed more particularly for animals fed at HI, whereas VFA molar proportions take these different aspects into account. Consequently, the individual regression equations for the following estimations of VFA production rates were calculated, for the two intake levels, from VFA proportions with the first four infusions (Con, B, Bp and bP) for the propionate, and with all the infusions (Con, B, Bp, bP and P) for the butyrate.

Estimation of volatile fatty acid production rates by the non-tracer and tracer method

Estimations of the rumen production rates of the different VFA from the propionate and butyrate with the NTM were comparable within the same intake level (C_3 *v.* C_4, $P > 0·05$, Table 5). However, the rumen production of the individual VFA differed between the two methods tested (NTM *v.* TM, $P < 0·001$, Table 5), irrespective of the intake level considered (method×intake level interaction, NS). Values were on average 1·5-fold higher when estimated by the TM compared with the NTM. The correlation ($P < 0·05$) between the two techniques for the estimation of the rumen propionate production rate was: NTM = 0·74 TM (SE 0·13) − 0·047 (SE 0·319), *r* 0·87 (Fig. 1). Regardless of the VFA considered and estimation method, the rumen VFA production rates, expressed in mol/d, differed between the two intake levels ($P < 0·001$, Table 5). The rumen production rate of total VFA was on average 1·7-fold higher for animals fed at HI (1550 g DM intake) than for those fed at LI (810 g DM intake). Rumen VFA production rate/kg DM intake was slightly higher for LI than HI, but the difference was not significant ($P > 0·05$).

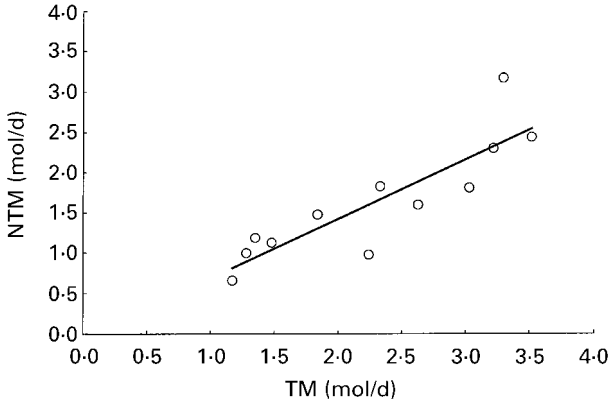

Fig. 1. Relationship ($P < 0·05$) between the non-tracer method (NTM) and tracer method (TM) for determination of propionate production rate in the rumen of sheep (*n* 12) fed two intake levels. For details of diets, infusions and procedures, see Table 1 and p. 332. NTM = 0·74 TM (SE 0·131) − 0·047 (SE 0·319), *R* 0·870.

Discussion

Effect of volatile fatty acid infusions on rumen variables and digestibility

The main assumptions common for both the tracer and non-tracer techniques for *in vivo* estimation of VFA production rates are that they cause no modifications of the rumen characteristics or of the microbial ecosystem, in order to avoid any disturbance in fermentation of the basal diet. In our study, unlabelled VFA infusions compared with the basal diet (Con) slightly decreased the liquid dilution rate for animals at HI, and increased both the rumen osmotic pressure, although values remained in the physiological range (Rémond *et al.* 1995), and the rumen pH. Addition of NaOH in the VFA solutions infused to adjust their pH to 6·0 might explain this effect of the rumen pH. Huhtanen *et al.* (1993) and Miettinen & Huhtanen (1996) did not find a significant effect of the VFA infusions on the rumen pH. However, in their experiments, the quantities of VFA infused were smaller compared with those used in the present trial (0·75 *v.* 1·35 mol propionate/kg DM intake), the pH of solutions was adjusted to only 4, which required relatively lower amounts of NaOH, and no treatment in which no VFA solution was infused (corresponding in our present study to the treatment Con) was included in their experimentation. It can be concluded that infusions of VFA did not involve significant changes in the microbial

Table 5. Estimation of rumen volatile fatty acid production rate (mol/d) by the non-tracer method (NTM) and tracer method (TM) in sheep fed two intake levels†
(Mean values with their standard errors for six sheep)

	High intake level			Low intake level				Statistical significance of effects			
	NTM			NTM				Method (M)			
	From propionate (C_3)	From butyrate (C_4)	TM	From propionate (C_3)	From butyrate (C_4)	TM	SE	NTM, C_3 *v.* C_4	NTM *v.* TM	Intake level (L)	Interaction (M × L)
Acetate	6·65	5·94	9·20	3·78	3·72	5·69	0·46	NS	***	***	NS
Propionate	2·19	1·95	3·01	1·07	1·03	1·56	0·15	NS	***	***	NS
Butyrate	1·45	1·30	2·04	0·78	0·77	1·18	0·10	NS	***	***	NS
Isobutyrate	0·107	0·095	0·149	0·077	0·074	0·115	0·008	NS	***	***	NS
Valerate	0·135	0·120	0·188	0·068	0·065	0·099	0·008	NS	***	***	NS
Isovalerate	0·100	0·089	0·140	0·081	0·078	0·121	0·007	NS	***	**	NS
Caproate	0·032	0·028	0·045	0·022	0·021	0·033	0·002	NS	***	***	NS
Total (mol/kg DM intake)	6·86	6·12	9·52	7·37	7·10	10·87	0·65	NS	***	NS	NS

† For details of diets and procedures, see p. 332.
** $P < 0·01$, *** $P < 0·001$.

ecosystem since the population of protozoa remains constant between treatments. It would appear, therefore, that the amount of VFA infused in our study had only minor effects on the rumen fermentation of the basal diet, and the comparable DM digestibility of the diet between treatments supported this conclusion.

Intake level

Irrespective of the estimation method and the VFA considered, the rumen total VFA production rate, expressed in mol/d, differed between the two intake levels, and was on average 1·7-fold higher when animals ingested 1·9-fold more feed. To our knowledge, no results are available in the literature about the effect of intake level on direct estimation of *in vivo* VFA production rate in the rumen. VFA production rates in the rumen of sheep have been estimated from rumen VFA concentration (Leng, 1970). Using the same equation, the total VFA production rate increased between HI and LI, although this difference was lower (1·4-fold) than found in our present study (1·7-fold). The rumen total VFA production rates, expressed per kg DM intake, were comparable between the two intake levels. This suggested that the rumen production of VFA was proportional to the amount of OM digested in the rumen. Hence, in the following discussion, results on the rumen VFA production rates will be expressed per kg DM intake and pooled over the two intake levels.

Volatile fatty acid production rate by the non-tracer method

Both estimation methods for the rumen VFA production rate were based on the assumption that molar proportions in the rumen represent ratios in production and thus that absorption rates of the different VFA were similar. One advantage of the NTM was that replacing butyrate infusion with propionate gave information on absorption rates of the different VFA. The rumen production rates of the individual VFA calculated from the propionate and butyrate with the NTM were comparable within the same intake level. This suggested similar fractional absorption rates between the different VFA and was in agreement with other results (Kristensen *et al.* 1996; Nozière *et al.* 2000). In addition, Dijkstra *et al.* (1993) observed equal rumen disappearance rates for acetate, propionate and butyrate at rumen pH in the region of 7, close to those (6·5) measured in our study.

The mean rumen production rates of acetate, propionate and butyrate with the NTM were 4·3, 1·3 and 0·9 mol/kg DM intake respectively. Assuming that OM digestibility in the total tract is 2 % higher than DM digestibility, and that OM apparently digested in the rumen is 0·75, we calculated the quantity of acetate, propionate and butyrate produced in the rumen using the stoichiometric principle (Demeyer, 1991). Values obtained were 3·5, 1·1, and 0·8 mol/kg DM intake for acetate, propionate and butyrate respectively and were close to those obtained in our study. Calculated as the sum of acetate, propionate and butyrate production rates, the total rumen VFA production rate in the rumen was 6·5 mol/kg DM intake and equivalent to 7·8 MJ ME/kg DM intake. Compared with the ME ingested (9·8 MJ ME/kg DM intake), the rumen VFA production corresponded in our

Table 6. Reported total volatile fatty acid production rates estimated by the isotope dilution technique for different animal species given various diets

	Experiment characteristics			Total volatile fatty acid production rate	
References	Animal	Diet	Method	mol/kg DM intake	mol/kg digestible organic matter intake
Bergman *et al.* (1965)	Sheep	Grass	$^{14}C_4$ infusion	7·8	10·5
Leng & Leonard (1965)	Sheep	Lucerne	$^{14}C_{2,3,4}$ infusion	10·3	19·1
Leng & Brett (1966)	Sheep	Lucerne	$^{14}C_{2,3,4}$ infusion	10·3	19·8
	Sheep	Lucerne–maize (25:75, w/w)	$^{14}C_{2,3,4}$ infusion	10·1	13·6
	Sheep	Lucerne–maize (50:50, w/w)	$^{14}C_{2,3,4}$ infusion	6·8	10·1
	Sheep	Lucerne–straw (10:90, w/w)	$^{14}C_{2,3,4}$ infusion	3·0	7·5
Weller *et al.* (1967)	Sheep	Hay	$^{14}C_{2,3,4}$ infusion	5·5	11·7
Nozière *et al.* (2000)	Sheep	Hay	$^{13}C_3$ infusion	6·1	10·4
Esdale *et al.* (1968)	Dry cow	Lucerne	$^{14}C_{2,3,4}$ infusion	7·6	14·9
	Dry cow	Maize silage	$^{14}C_{2,3,4}$ infusion	7·9	11·8
Oshio *et al.* (1977)	Heifer	Hay–concentrate (60:40, w/w)	$^{14}C_2$ pulse dose	4·4	6·8
	Heifer	Hay–concentrate (70:30, w/w)	$^{14}C_2$ pulse dose	5·3	8·5
	Heifer	Concentrate	$^{14}C_2$ pulse dose	4·8	5·9
Sharp *et al.* (1982)	Steer	Hay–whole maize (16:84, w/w)	$^{14}C_{2,3,4}$ infusion	9·2	13·9
	Steer	Hay–ground maize (16:84, w/w)	$^{14}C_{2,3,4}$ infusion	7·1	10·8
Siciliano-Jones & Murphy (1989)	Steer	Hay–concentrate (80:20, w/w)	$^{14}C_{2,3,4}$ infusion	7·0	12·1
	Steer	Hay–concentrate (20:80, w/w)	$^{14}C_{2,3,4}$ infusion	6·9	9·1

present study to 79 % ME intake and appeared to be a reasonable estimate, even probably slightly overestimated compared with values (65–75 % total ME) reported by Bergman (1990) in his review. Hogan & Weston (1967) reported a close linear relationship between OM digested and total VFA rumen production rate in sheep fed *ad libitum*, with a slope of 9·2 mol VFA/kg OM digested. This value was similar to that measured in the present study (10·0 mol VFA produced/kg OM digested).

Volatile fatty acid production rate by the tracer method

With the TM, the total VFA production rate (sum of acetate, propionate and butyrate production rates) averaged 9·8 mol/kg DM intake. In the literature, the total VFA production rate obtained by the isotope-dilution technique was on average 7·1 mol/kg DM intake, but the range of variation was high and values varied from 3·0 to 10·3 mol/kg DM intake (Table 6). To take into account variations due to the nature of the diet, we expressed our results and those of the literature per kg of digestible OM intake. In our experiment, total VFA production rates were 15·6 mol/kg digestible OM intake and were included in the range of total VFA production rates observed in the literature (from 5·9 to 19·8 mol/kg digestible OM intake, Table 6). In terms of ME, estimated rumen VFA production was equivalent to 11·8 MJ ME/kg DM intake and represented 120 % total ME intake, suggesting an overestimation of the rumen VFA production rate with the TM.

The rumen production rate of individual VFA was on average 1·5-fold higher when estimated by the TM compared with the NTM. Only one other direct comparison between these two techniques for measuring the VFA production rate can be found in the literature (Nozière *et al.* 2000). In that study, ewes were fed a low level of intake (500 g DM intake), and the higher rumen propionate production rate (1·5-fold) with the TM compared with the NTM was similar to our results (1·5-fold). An eventual interconversion of the labelled propionate to the other VFA may explain the discrepancy between the two estimation methods for rumen VFA production rate, but this does not agree with the literature (Bruce *et al.* 1987; Peters *et al.* 1990), stating that the exchange of propionic acid C with other VFA is quantitatively insignificant. A possible sequestration of VFA by the rumen micro-organisms for biosynthesis, which may cause overestimation of the VFA production rates with the TM, has been suggested (Kristensen & Danfaer, 2000), but this has never been quantified *in vivo* for propionate. Concerning the metabolism of acetate by rumen micro-organisms, quantitative estimates made *in vitro* (Emmanuel *et al.* 1974) and *in vivo* (NB Kristensen, personal communication) indicated that only 3 % and 8 % of the labelled acetate C were incorporated into the microbial biomass respectively. Thus, the more probable reason for the overestimation of VFA production rates with the TM may be related to the position of the labelled C on the propionate ([1-^{13}C]propionate). Indeed, in his review Sutton (1985) reported problems of overestimation of propionic acid production when [1-^{14}C]propionate was used rather than [2-^{14}C]propionate due to the labile nature of the carboxyl-C transformed into $^{14}CO_2$ in the rumen.

Irrespective of the method used, a comparable effect of intake level on the rumen VFA production rate was observed. The production of VFA in the rumen increased proportionally (1·7-fold) to the amount of OM ingested by the sheep for intakes increasing to 0·9–1·7 times maintenance. According to the accuracy of techniques (for the propionate, SE 0·42 mol/d for both methods), the TM and NTM are suitable to show differences in VFA production rates between diets characterized by very different OM digestibility. This variability in the measurements with the

different methodologies might make more difficult statistical comparisons for intermediates diets.

The two techniques tested provide different rumen VFA production rate estimations. According to ME intake, rumen VFA production rates with the NTM appeared reasonable, but the NTM requires at least three rates of VFA infusion, which makes it laborious. On the other hand, the TM appears easier to apply and the small quantity of labelled VFA infused limits risks of unphysiological situations. However, the TM seemed to overestimate the rumen VFA production rate. The potential reasons for this overestimation with the TM were proposed and are being investigated in further studies.

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