CHAPTER 4

ESTIMATION OF RUMINAL METHANE PRODUCTION FROM MEASUREMENT OF VOLATILE FATTY ACID PRODUCTION

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INTRODUCTION

Reduction of carbon dioxide to methane is one of the terminal steps in electron transfer within the network of fermentation reactions conducted by rumen micro-organisms. Clearing the rumen of hydrogen gas by methanogenesis allows ongoing regeneration of NAD^+ and FAD^+ and the continuous conversion of dietary carbohydrate and protein into microbial cells and volatile fatty acids (VFA). Given this mechanistic link between fermentation and methanogenesis, it is not surprising that stoichiometric associations based on hydrogen recovery have been considered to be the basis for linking methane production and VFA production data (8, 16, 26). The association is quite strong when fermentation occurs in a normal manner but is uncoupled when methane inhibitors are included, presumably because alternative hydrogen pathways are induced.

This chapter describes in three sections the technical procedures associated with infusing or injecting ^{14}C-labelled VFA into ruminants, the mathematical procedures for calculating VFA production rates and the stoichiometric relationships that allow methane production rate to be predicted from VFA production rates. A critique of this approach is also included.

1. MEASURING VOLATILE FATTY ACID PRODUCTION RATE IN THE RUMEN BY ISOTOPE DILUTION

1.1. Principle of Method

VFA labelled with isotopes can be used as tracers to estimate the rate of VFA production in the rumen. A known quantity of VFA tracer is injected or infused

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into the rumen and the dilution over time of the labelled VFA (tracer) by unlabelled VFA (tracee) arising from fermentation is determined (this method is referred to as the tracer dilution method). The most widely used tracer methods involve the continuous infusion of ^{14}C- or ^{3}H-labelled VFA either singly or as mixtures of VFA, with samples of rumen fluid being taken at intervals over a period of several hours (19, 12). The *net flux* of VFA (mol/min) through the compartment into which a tracer is infused is calculated by dividing the rate of infusion of radioactive isotope (MBq/min) by the concentration of radioactivity in the VFA (MBq/mol) isolated from the rumen fluid samples. A more complete understanding of the rates of production of individual VFA and their interconversions can be obtained by solving multi-compartment models (e.g. 22, 23).

A general three-compartment model showing the total fluxes of acetate, propionate and butyrate through their respective compartments from internal and external sources and the interconversions of tracee is shown in Figure 1.

The terminology used to describe the flows of VFA in a model such as the one shown in Figure 1 can be a cause of confusion. It is important to focus on the *tracer* being used. If a ^{14}C- or ^{13}C-labelled VFA is used as the tracer, then the material being traced (or *tracee*) is carbon. If ^{3}H or deuterium is the label, then flow rates refer primarily to the flows of hydrogen. The estimated flow rates are fluxes of the tracee through the kinetic *compartments* that comprise the model, e.g. acetate, propionate and butyrate in rumen contents. For the acetate compartment, the rate of *total flux* of tracee is given by the sum of all inflows into the acetate compartment which, if the rumen VFA system is in *steady state* will be the same as the sum of all outflow rates. This value is greater than the rate of *net flux* of acetate-C because, during a continuous infusion of labelled acetate, some of the acetate tracer will leave the acetate compartment and be converted to other materials (e.g. butyrate) and then subsequently be returned to acetate, i.e. *recycled*. Net flux rate has been referred to as *irreversible loss rate* by some workers. The

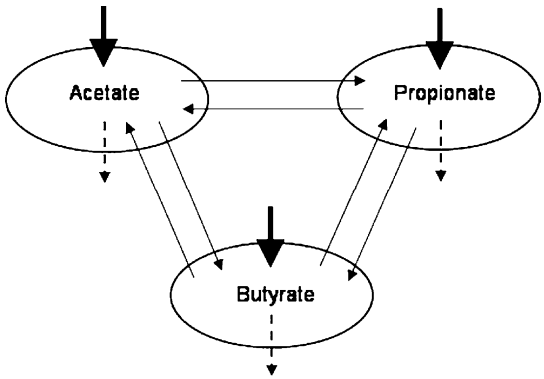

Figure 1. An open, general, three-compartment model describing all possible carbon fluxes into, from and between each of the three major volatile fatty acids in the rumen

net flux rate (g C/d), which excludes this recycled tracer, is calculated by dividing the ^{14}C-acetate infusion rate (MBq/d) by the plateau specific radioactivity (SR) in acetate (MBq/g C). The compartment size, total flux rate and recycling rate are not readily determined by means of a *continuous infusion* of labelled VFA, but can be determined if a *single dose* of tracer is administered at one time and the SR versus time curve thereafter is analysed. Despite this apparent advantage, the single dose method has other limitations that will be discussed later.

The above general three-compartment model (Fig. 1) can be solved if three separate experiments, each involving the continuous infusion of one of the three acids containing labelled carbon, are performed. The plateau SR values for each compartment are determined at known rates of tracer infusion in each experiment, giving 9 plateau SR values and 3 infusion rates. These values enable all the flow rates in the model to be quantified. Several more terms can be defined. Each compartment has a flow of material into it from outside the model, and a flow from that compartment to the outside. The first represents the rate of incorporation of tracee (new or *de novo* production of VFA; bold solid arrows in Fig. 1) and the second represents tracee that is permanently removed from the model system (*final disposal rate*; bold dashed arrows in Figure 1 which reflects VFA available for absorption from the gut).

There is no ideal isotopic tracer for estimating the rates of VFA production. Tracer VFA can be labelled using radioactive or stable isotopes. Rates of net flux of the major VFA (acetate, propionate and butyrate) can be estimated individually using separate tracers for each VFA. Alternatively, one labelled VFA can be used and a good estimate of the combined rates of net flux of all VFA obtained (34, 35). Production rates of the individual VFA can be predicted reasonably accurately from their molar concentrations in rumen fluid (20).

Prediction depends on the linear relationship that exists between net production of an individual VFA in the rumen, expressed as a fraction of total VFA production, and its concentration, expressed as a fraction of the total VFA concentration (19, 20). However, Weston and Hogan (35) and Rowe (27) suggest that a single VFA infusion gives unreliable estimates of the 'effective production rate' of individual VFA because factors affecting absorption rate are not well understood and there are variable rates of interconversion of carbon between the individual VFA.

As little propionate is metabolized to either acetate or butyrate in the rumen, ^{14}C-labelled sodium propionate is nearest to being an ideal tracer in experiments using only a single labelled VFA, but it is also the most expensive of the three ^{14}C-labelled VFA. As a less expensive alternative, 1-^{14}C, 2-^{14}C or uniformly labelled (U-^{14}C) acetate can be used to determine acetate (or total VFA) net production rate. Acetic acid is the predominant VFA produced by ruminal fermentation and is closely associated with ruminal hydrogen production. Acetate interconverts with butyric acid and this interconversion can cause underestimates of acetate production by 6% if not allowed for (15). This error can be overcome by making separate injections or infusions of labelled acetate and butyrate on separate days to quantify the interconversion. These complexities are explained in Section 2.

Procedures for estimating VFA production in ruminants *in-vivo* are described in detail below. They apply mainly to experiments to be made in rumen-cannulated sheep or goats but additional notes for cattle studies are provided where needed. Considerable planning is required before an infusion experiment is undertaken but, with good planning, one scientist can comfortably conduct injections or infusions and take samples from 6 sheep simultaneously.

While there is no maximum time for infusions, a 9 h infusion, with sample collection over the final 6 h can give a good estimate of daily production if animals are given their daily ration in equal hourly portions. The 6 h period of sampling is long enough to allow averaging of small variations in VFA production rate that may occur as the substrate supply for the rumen microorganisms varies between meals.

1.2. Preparation of Animals

1.2.1. Materials

- Rumen cannulated sheep or cattle
- Automatic feeders delivering feed at regular intervals
- Peristaltic infusion pump and flow-rated pump tubes
- Infusion tubing (Silastic; 1.5 mm i.d.)
- Ruminal probe with infusion and sampling tubes

1.2.2. Procedure

Animals should have been fitted with cannulas at least 4 weeks prior to VFA production being measured and should have been held in the experimental housing for at least two weeks to allow them to adjust to their experimental diet and surroundings. Animals should be housed in metabolism cages or tethered so that they are not able to rotate in the pen, which can cause infusion lines to twist and fail. The area around the animals should be well ventilated, especially if radioactive tracers are to be used, to prevent the accumulation of hazardous concentrations of radioactive end-products in the area. The diet should be provided in equal portions at regular frequent intervals in the 7 days leading up to, and throughout, the infusion period. Ideally, the diet should be provided in equal hourly portions by an automatic feed-dispensing machine fitted with a 24 h timer. Animals should also be accustomed to their attendants and the procedures for taking fluid samples. Unaccustomed animals may become stressed and refuse to eat during the period of VFA measurements, which will compromise the results obtained.

1.3. Preparation of Isotope Stock and Infusion Solutions

1.3.1. Materials

- Isotope source (supplied in glass ampoule or screw-top bottle)
- Tray (20 cm × 30 cm) with sides and absorbent liner to contain isotope during transfer

- Pasteur pipettes
- Latex gloves
- Tissues
- Water
- 10 M NaOH (< 10 mL)
- 20 mL McCartney bottles and lids
- Radioactive waste disposal bag
- 10 mg of non-radioactive sodium salt of the VFA

1.3.2. Procedure

Volatile fatty acids are normally purchased as the sodium salt (> 99% chemical purity) and isotopically labelled on one or more carbons. Deuterium label (at least in acetic acid) exhibits exchange with other hydrogen atoms in the rumen so is not used (18) although tritiated butyrate is used (19). During preparation of the isotope stock solution, the operator should wear disposable latex gloves to prevent contact with the highly radioactive β–emitting ^{14}C isotope. The ampule containing the dried down VFA salt can be dissolved in water and transferred into a thick walled glass bottle (20 mL) as follows (Fig. 2).

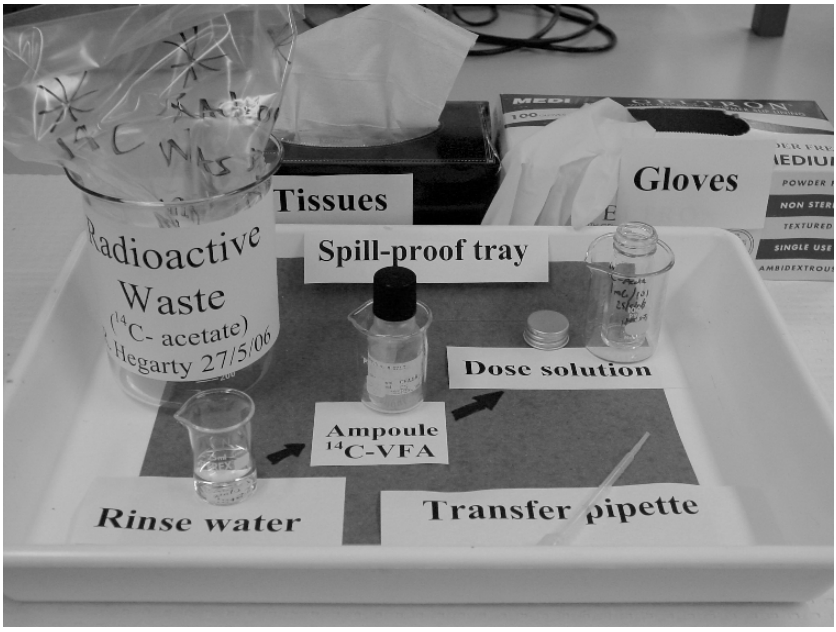

Figure 2. Tray for preparation of radioisotope stock solution. The tray has raised edges to prevent spillage, is lined with absorbent paper and has a waste disposal bag. The vial containing the isotope to be opened, together with transfer equipment (pipette, wash solution) and a labelled bottle to receive the isotope solution are present. Tissues are present in case of spillage and to help safely snap the ampule when tracers come as powders in ampules

- Line a waterproof tray having raised edges with absorbent paper.
- Place in tray:
  - Ampule, sitting upright in 50 mL glass beaker
  - File/scratcher to score ampule
  - Pasteur pipette with functional rubber bulb
  - Tissue to place around scored ampule to contain glass when snapping scored neck of ampule
  - Open-topped bottle (scintillation vial) containing 10 mL of distilled water into which 10 mg of the sodium salt of the VFA had been dissolved (this non-radioactive 'carrier' is added to prevent the radioactive acid binding extensively to glass and infusion lines)
  - Empty 20 mL McCartney bottle. This bottle should be labelled 'STOCK' or 'DOSE SOLUTION' and have all details of the isotope solution it will contain written onto it with a permanent marker. The sticker adhering to the ampule or its packaging can also be removed and stuck to this dose bottle.
  - 1 L glass beaker lined with plastic bag for waste. This should be labelled with your name, ^{14}C waste, a date and 'Caution radioactive waste' written in large visible letters.
- Using the file, score the neck of the ampule at the narrowest place, then wrap the scored section in tissue before snapping the ampule at the score mark. Dispose of tissue into waste bag.
- Draw approximately 1 mL of water into the pasteur pipette and dispense into the open ampule. After swirling the ampule gently, all the VFA salt should dissolve. The solution can then be drawn back into the pasture pipette and dispensed into the labelled McCartney bottle.
- A further 9×1 mL washes of water can be transferred into the ampule to wash it and then drawn back out and placed in the McCartney bottle being used for storage.
- The final step in preparation is to add 0.1 mL of 10 M NaOH to make the stock solution alkaline, screw the lid on the stock solution and place in freezer until needed. NOTE: To prevent cracking during freezing, the bottle should not be more than $^3/_4$ full and should be allowed to cool and freeze slowly.
- The empty ampoule, the tissue/s, the small bottle that originally held water and the pasture pipette and the paper lining the tray can all be placed in the radioactive waste bag. This bag should be disposed of according to nationally accepted procedures.

From this stock solution of isotope, working solutions for intraruminal injection or infusion can be prepared and utilized as described below.

1.4. Intra-Ruminal Infusion of a ^{14}C-Labelled VFA

The amount of activity infused (MBq/min) is the product of the activity in the infusion solution (MBq/mL) and the rate of infusion (mL/min). In sheep, infusions are typically made at a rate of 0.6–0.8 mL/min and for this rate, the infusion solution

should be prepared by dilution of the dose solution to contain 11.1×10^3 Bq/ml (0.3 μ Ci/mL). For cattle the infusion rate is higher (10 mL/min) and the radioactivity content (Bq/mL) in the infusion solution similar. Alkali (0.7 mL of 10 M NaOH per litre of infusion) should be included to prevent microbial growth and volatilization of VFA. Infusion solutions should only be prepared on the day prior to infusion and held under refrigeration prior to use.

1.4.1. Materials

- Dual inlet rumen probes (Fig. 3)
- Silastic tubing (1.5 mm i.d.). This must be at least 1 m longer than the distance between pump and sheep
- Short (60 cm) lengths of silastic tubing to conduct infusion solution from infusate bottles to pump
- Woollen thread
- Scales accurate to 1 mg
- Clock/timer
- Bottles or Erlenmeyer flasks containing the infusion solution. One bottle per sheep plus one bottle of 'spare' infusate per pump used. These must be numbered with the number of the animal/pen to which the infusion is being made (Fig. 4)
- Peristaltic infusion pump fitted with correct flow-rate pump tubing
- Tray lined with absorbent paper to house pump
- Connectors to join pump to inlet and outlet lines and join infusion line to rumen probe

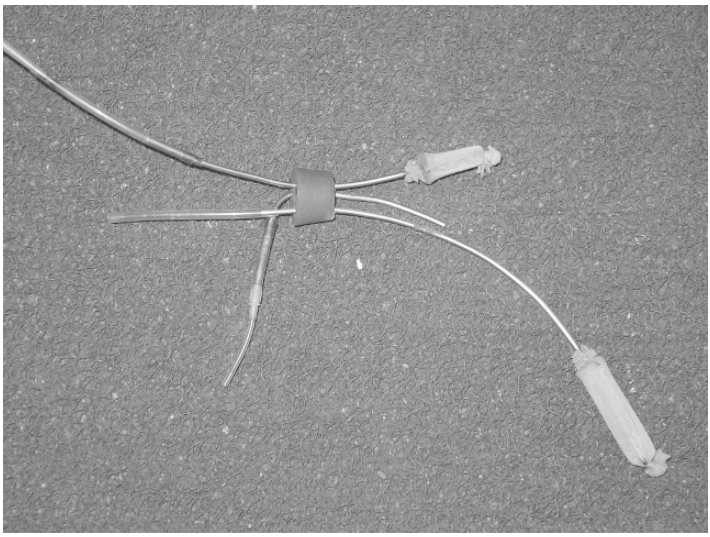

Figure 3. Rumen probe showing positioning of isotope infusion probe (middle) relative to sampling probes for rumen fluid (lower) and headspace gas (upper)

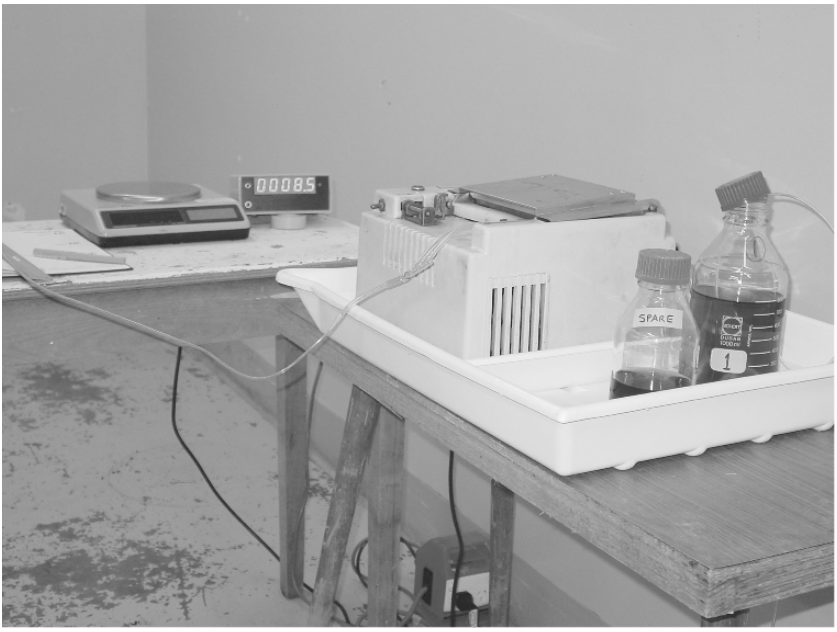

Figure 4. Infusion pump set up for intraruminal infusion. The plastic tray contains the peristaltic pump, an infusion bottled labelled with animal number, a spare bottle of infusate. Scales to determine the weight of infusion bottle, a clock and a data book to record weights, times and comments are nearby

- Infusion solution
- Rumen fluid collection bottles (stock solution) labelled with animal and sample number. Approximately 8–10 bottles will be required per animal. Each bottle should be prepared with 0.3 mL of 18 M H_2SO_4 as a preservative before the rumen fluid is added.

1.4.2. Procedure

Assuming animals have been eating as required, have recovered from cannulation and are tethered or caged so they cannot turn in circles, a 9-h ^{14}C-VFA infusion can be conducted as follows:

- Place peristaltic pump in tray on a table at a close but safe distance from the animal.
- Connect short (60 cm) inlet line to the inflow side of pump and long infusion line from pump outlet reaching to the sheep. This tube will need to have flexibility to permit the animal to stand or lie down and move in the metabolism crate. This can be set up some days before the infusion is to happen.
- The infusion line should preferably be suspended from above and behind the sheep (to avoid it being chewed), and be tied to tufts of fleece above the tail and again near the rumen cannula (Fig. 5). This prevents the infusion line pulling

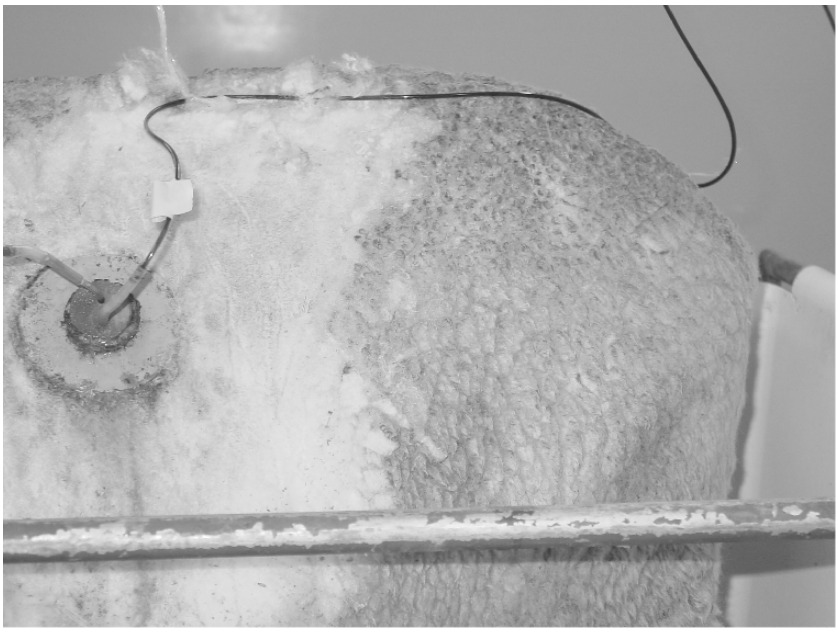

Figure 5. Infusion of ^{14}C-VFA and CrEDTA in progress. The infusion line is tied to the wool near the cannula and near the tail to prevent the infusion line being pulled loose. The infusion line is hung from the ceiling using a chain of linked rubber bands to allow the animal to stand up or lie down without the line going slack and being damaged

from the cannula when the animal lies down. A system for keeping the tube taut above the animal as it moves in the cage should be devised (e.g. suspending the infusion tubing from the ceiling using a length of elastic).

- On the day of the infusion, the rumen probe should be inserted in the rumen and a background sample(s) of rumen fluid collected before tracer is administered. It is important that the inlet probe should reach deep into the rumen contents, while the sampling probe should have its intake at least 15 cm from the infusion outlet. This is essential as it must be assumed that the sample taken is representative of the whole rumen contents at that time and the infusate has mixed completely in the rumen. The rate of mixing of rumen contents is a major infusion issue as discussed subsequently.
- Subsamples of the infusion solution MUST be taken into acid-free clean, fully labelled bottles (McCartney) at least before and at completion of the infusion. These should be stored frozen with all samples collected. No sample is more important than the sample of infusion solution!
- The rate of infusion is determined from the loss in weight of infusate over the period of infusion. Record the full (starting) weight of each animal's infusion bottle immediately before commencement and record in data sheet (worked example in Table 1).

Table 1. Data Recording Sheet to Monitor Rate of Infusion (g/Min) by Change in Weight of Infusate Reservoir During Intraruminal Infusion of Labelled VFA. A Separate Sheet Should also be Prepared to Record the Time at Which Each Sample from Each Animal is Collected

Date	16/02/2005			
Infusion	1–^{14}C acetate (Na salt) approx 0.25 μCi/mL			
Diet	500 g lucerne + 100 g barley			
Real time infusion started	07:52 h			
Sheep	1	2	3	4
Bottle weight start (g)	1526.21	1725.91	1550.16	Not eating
Bottle weight end (g)	1350.94	1553.22	1390.88	Not done
Weight change (g)	*175.27*	*172.69*	*159.28*	X
				X
Time start (by digital clock)	0012	0012	0013	X
Time end	0220	0220	0222	X
Duration (min)	208	208	209	X
Infusion rate (g/min)	***0.8426***	***0.8302***	***0.7621***	
Weight and time cells for repeat measures below				

- Commence the tracer infusion by connecting the infusion line to the rumen probe and then turning on the infusion pumps and recording the "start time" from the clock onto the data sheet.
- No samples will be collected during the first 3 h of infusion.
- After 3, 6 and 9 h of infusion, the bottles of infusion should be weighed and the time and weight recorded on the data sheet. While the bottles are being weighed, the intake lines leading to each pump can be transferred to the spare bottle of infusate to ensure the infusion into animals is not interrupted. When the bottles have been weighed, the intake lines can be quickly swapped back into the infusion bottle for each sheep. These measurements allow three three-hourly measures of infusion rate (g/min) to be obtained for each animal and help identify pump failure if it occurs.

1.4.3. Sample collection and storage

- After 3 h of infusion, samples of rumen fluid can be collected at approximately 50 min intervals.
- Rumen fluid samples should be taken after drawing fluid into the syringe (25 mL) and either discarding or re-injecting this fluid into the rumen at least three times. This ensures the sample is fresh fluid from the rumen digesta. Some frothing of sample may occur due to the H_2SO_4 already in the sample bottle so the 15 mL of sample should be dispensed slowly into the sample bottle.
- Samples should be sealed and frozen on collection.

All tracer dilution methods are based on assumptions about kinetic compartments. By definition, a compartment is a biological entity within which the movement of materials is relatively rapid when compared with movement to and from areas outside the compartment. This means that the boundaries of the compartment effectively restrict the movement of the material of interest.

For accuracy of estimates of VFA flux rates from infusion (or single injection) studies there must be a rapid and uniform mixing of labelled VFA throughout the entire reticulo-rumen. This requirement is seldom ideally met. Variations in VFA concentrations can occur in different sites within the reticulo-rumen indicating that the rumen contents are not always homogenous.

Slow or incomplete mixing of tracer can be a major source of error when tracer techniques are used to estimate VFA production rates. In recognition of the importance of mixing in the compartment into which tracer is administered, researchers have tried many options for improving the rate of tracer mixing. For example, Sutherland *et al.* (31) and Blake *et al.* (3) devised pumping systems that artificially circulated rumen contents to achieve more rapid mixing of tracer and tracee. To assist the rate of distribution of tracer through the rumen contents, the rate of infusion of solution containing the tracer should be as high as possible – with a balance being struck between the volume added and the extent of perturbation of normal water kinetics in the rumen. To improve tracer and tracee mixing in the rumen ammonia compartment during a continuous intraruminal infusion experiment using labelled ammonium chloride, Oldham *et al.* (25) placed a stainless steel frame in the rumen on which there were two tracer infusion sites approximately 20 cm apart, sited near the centre of the rumen, and three separate sampling tubes. The frame was designed to improve the distribution of tracer in the rumen contents and also to shorten the time taken for tracers to become evenly distributed throughout the rumen contents, and to keep a discrete distance between the points of infusion and the points of sampling.

To help workers to recognize and allow for problems of less-than-ideal mixing of tracer, we recommend that a liquid marker (such as Cr-EDTA or Co-EDTA) is included in the infusate containing the labelled VFA. The Co or Cr kinetics indicate how quickly and uniformly the infusion solution mixes throughout the rumen contents.

1.5. Single Ruminal Dose (Injection) of ^{14}C Labelled VFA

An alternative to infusion is to make a single injection of a more concentrated ^{14}C-VFA into the rumen. Sheppard *et al.* (29) and Gray *et al.* (12) used single injections of ^{14}C-labelled VFA to estimate their production rates in the rumen of sheep. An instantaneous injection offers several advantages over infusion, *viz.*

- problems associated with infusion pumps and broken infusion lines are avoided,
- the injection can be made at multiple sites within the rumen assisting the more rapid and even distribution of the tracer in the rumen contents, and
- the isotope dilution curve allows compartment size of the VFA and total flux rate to be determined, not just net flux rate.

A disadvantage of single injection experiments is that the production rate estimates apply to the few hours after injection which may not give a good estimate

of daily VFA production rate. The method also requires that samples are taken more frequently during this critical time.

The conducting of an intraruminal ^{14}C-VFA injection should be as follows:

- Dose prepared of 50 mL × 56 kBq/mL and containing 25 mL of Cr-EDTA solution (1).
- Sub-samples of injection solution taken into labelled empty McCartney bottle and stored frozen (do not fill > ¾ full).
- Dose dispensed into 50 mL syringes and full-syringe weighed immediately prior to injection.
- Dose introduced via 40 cm (× 3 mm i.d.) curved steel tube and rapidly dispensed into 10 rumen sites with approximately 5 mL/site.
- Dose syringe removed and new syringe with 100 mL of water used to wash residual dose from steel tube into the rumen.
- Final (empty) weight of injection syringe MUST be determined to enable the weight of dose injected to be determined.
- Time of dose introduction and time of each individual sample must be recorded precisely.
- Samples taken 20, 40, 60, 90, 120, 150, 180, 240, 300, 360, 420 min post-injection and stored in acidified McCartney bottles and frozen.
- If more than one ^{14}C-VFA infusion or injection is to be conducted, the preferred dosing order is butyrate, propionate then acetate and if possible, a day should be allowed between each infusion to allow residual radioactivity to be diluted out.

1.5.1. Sample Analysis

The fundamental analytical requirement for this or more complex analyses is to establish the specific radioactivity (SR) of the infused VFA in the rumen, *i.e.* radioactivity (MBq per mole of VFA). This requires the VFA of interest be isolated, quantified and its radioactivity determined.

In early studies, Gray *et al.* (12) simply steam distilled rumen fluid to release all VFA and determined the concentration of radioactivity in the distillate after determining the total VFA present by titration. This method is still used by some workers (30). Weller *et al.* (34) subsequently showed that the SR of a single labelled VFA could be used to give satisfactory estimate of the net flux of all three major VFA. There are a number of means by which radioactivity in rumen contents can be obtained including:

- Quantification and isolation of labelled VFA by HPLC and determination of radioactivity by liquid scintillation counting (e.g. 13).
- Quantification and isolation of labelled VFA by liquid chromatography and determination of radioactivity by liquid scintillation counting (e.g. 19).
- Gas chromatographic quantification of component VFA and on-line determination of radioactivity by means of a proportional gas flow through counter. Czerkawski and Breckenridge (1975) utilized this radioactivity sensor system but it has not been widely used. The simplicity of the system and low cost compared to liquid chromatography and liquid scintillation could justify its renewed investigation for application in developing countries.

2. CALCULATION OF VFA PRODUCTION FROM SAMPLE SPECIFIC RADIOACTIVITY

Several calculation options are presented. The analysis to be used will depend on whether the results are derived from single dose experiments, or single or multiple infusion experiments. In all cases, VFA samples taken before tracers were administered should be assayed for background radioactivity and the results subtracted from VFA samples taken after the administration of tracer. Most analyses of the data involve certain assumptions, and the analyses in common use often do not use all of the information implicit in the SR versus time curves resulting from tracer dilution.

2.1. Single Dose Experiments

These are studies in which a single bolus of ^{14}C-VFA is injected into the rumen at a single time and the dilution of added radioactivity followed over 6–9 h. One limitation is that instantaneous mixing of tracer and tracee is assumed. Also VFA production in the period soon after injection contributes more to the flux estimate than production later in the sampling period.

- If a liquid marker is introduced with the ^{14}C-VFA tracer, the concentration of marker in all samples (mg Cr-EDTA/L) should be analysed. If the decline in marker concentration with time is well fitted by a single exponential function, this indicates there was rapid mixing of the labelled solutions and a steady state of fluid turnover in the rumen existed. If early data points vary from the line of best fit, the implication is that the injection solution was slowly distributed throughout the rumen contents.
- The rumen fluid volume is given by dividing the dose of Cr-EDTA injected (g) by the intercept of the curve at time zero (g Cr-EDTA/L rumen fluid). This information, together with values for the molar concentrations of individual VFA, can be used to estimate the VFA compartment sizes (g C) in rumen contents.
- Assuming mixing of the dose solution was deemed satisfactory, the SR versus time curve should be fitted using single or multi-exponential functions (Eqn. 1), depending on the tracers used (22).

$$(1) \qquad SR_t = \sum A_i \exp(-^{k}i^{t}) \qquad \text{(illegible)}$$

where SR_t is the SR at time t, A is the intercept of the curve at time zero and k_i is the rate constant of the i^{th} exponent.

- If propionate is infused, the propionate flux rate can be closely approximated from a single exponential decay whereas if acetate or butyrate were infused, where interconversions and recycling of ^{14}C will have occurred, then a multi-compartment model must be assumed and two or more components must be fitted to the curve as described above (Eqn. 1).

At time zero (the moment of injection) the multi-exponential equation simplifies to

$$SR_0 = \text{Sum } (A_i) \quad (2)$$

The exponents (A_1, $A_{2\ldots}$ A_i) are the intercepts of the component vectors that can be summed to give the SR at the moment of injection. Dividing the dose (Bq) by this SR (Bq/g C) gives an estimate of the size of the VFA compartment (g C).

The total flux of VFA carbon (TF, g C/d) and net flux of VFA (NF, g C/d) are given by

$$TF = \text{Compartment size (g C)} \times \text{(tangent to curve at time zero)} \quad (3)$$

$$NF = \text{Dose injected (Bq)/(area under curve (Bq/g C.d)} \quad (4)$$

(see Nolan and Leng (22)).

2.2. Continuous Infusion Experiments

- Again the background radioactivity in the pre-infusion samples of VFA should be subtracted from the radioactivity in all samples taken after the start of the tracer infusion.
- The SR (Bq/g C) versus time curve should be plotted to demonstrate that a constant SR had been reached after 3 h.
- A plateau SR can either be calculated as the mean of measured SRs from, say, 3–9 h after the start of infusion (assuming there is no trend in SR), or can be calculated after fitting an appropriate model to the data and predicting the SR at plateau.
- The net flux of the VFA through the primary compartment is calculated as follows:

$$\text{Net flux (g C/d)} = \frac{\text{Infusion rate (Bq/d)}}{\text{Plateau SR (Bq/g C)}} \quad (5)$$

The use of the net flux equation (Eqn. 5.) is a standard method for deriving the net flux of a tracee when only one acid is infused. However, various different ways of analysing results from one or more ^{14}C-VFA infusion have been used, producing different measures of VFA production. These options and their implications for estimates of methane production are discussed below. Differences in estimates arise depending on whether cycling of VFA carbon (between acetate and butyrate) is ignored or allowed for, and whether radioactivity is determined in individual VFA (e.g. SR of acetate-C) or in the bulked VFA (radioactivity in total VFA carbon).

The most detailed analysis of VFA production will be a complete analysis of the flows depicted in the general three-compartment shown in Figure 1. The model can

be solved by matrix algebra using Microsoft Excel™or other software (Excel version available on request). If several continuous infusions of tracer have been used, the data required are plateau SR values for acetate, propionate or butyrate during individual infusions of each acid, and the tracer infusion rates. It is assumed that the same conditions (animal diet and steady state etc) applied to each experiment.

To illustrate the range of possible combinations of ^{14}C-VFA tracer application in the animal, and laboratory analysis procedures, three approaches to analysing and interpreting VFA production studies are considered with respect to their effect on estimated methane production. Data of Bergman *et al.* (2) are used as they represent a comprehensive database that can be drawn on selectively to evaluate all data options. These workers infused labelled acetate, propionate and butyrate separately into sheep offered grass cubes continuously from a moving belt feeder (900 g/d). The rumen contents were artificially mixed with a pump. The total VFA concentration in rumen fluid was 105 mmol/L, acetate, propionate and butyrate concentrations expressed in molar percentages were 68:19:13 and compartment sizes of 395, 110 and 75.6 mmol or 9.49, 3.98 and 3.63 g C, respectively.

All production parameters for each VFA were derived from complete solution of the 3 pool model (total, net flux, *de novo* synthesis and VFA disposal) and stoichiometry used to predict the anticipated net hydrogen synthesis and methane (Approach 1). Data were then re-analysed to provide stoichiometric estimates of methane production had only the SR of a single infused VFA been known (acetate or propionate) (Approach 2) and from the gross SRs of the combined VFA together being measured after steam distillation (Approach 3).

Approach 1. *Infusion of radioactively labelled acetate in one experiment, propionate in another experiment and butyrate in a third experiment in sheep maintained under similar conditions throughout the three experiments*

This most costly and lengthy procedure, requires separate infusions of three individual ^{14}C-VFA tracers and determination of the SR of all three VFA during each experiment (e.g. 2, 17). This 3 compartment model gives estimates of total flux, net flux and *de novo* production rates for each compartment and requires as data inputs, VFA infusion rate in each experiment (mBq/d) and the SR at plateau (Bq/g C) for each of the VFA during each experiment. The infusion rates in each experiment (adjusted to 100 μCi/h) and the corresponding plateau SR values for Bergman *et al.* (2) are given in Table 2.

The general three-compartment model can be solved by solving 12 simultaneous equations describing the flows of tracer and tracee, or by a matrix algebra procedure. The model solution with all fluxes (g C/d) is given in Figure 6. The measures of VFA flux and methane production are summarized in Table 3.

The difference between total flux and net flux rate for each VFA compartment is an estimate of the rate of carbon recycling to the compartment. From Figure 6 and Table 3 it can be noted that:

- Use of total flux gives the highest estimate of methane production. This is because total flux includes carbon of the specific VFA that has been changed to some

Table 2. Plateau Specific Radioactivity of Rumen Acetate, Propionate and Butyrate in Sheep During Infusions of Labelled Acetate, Propionate and Butyrate in Separate Experiments. Results are Means From 3 Separate Infusions of Each of Three VFA Tracers and are SRs are Adjusted to Infusion Rates of 100 μCi/d Bergman *et al.*, (1965)

Tracer	Infusion rate (adjusted)	Acetate SR μCi/g C	Propionate SR μCi/g C	Butyrate SR μCi/g C
[1–^{14}C]-acetate	100 μCi/h	21.1	2.42	9.42
[2–^{14}C]-propionate	100 μCi/h	3.00	54.3	2.17
[1–^{14}C]-butyrate	100 μCi/h	12.8	2.58	47.5

other compound and back to that VFA during the experiment. Total flux should not be used to estimate methane production because hydrogen production or consumption need not be associated with remaking the VFA.

- The rate of net flux (also referred to as irreversible loss rate) from each VFA compartment is intermediate between the total flux and the rate of *de novo* synthesis (bold arrows) or final disposal (dashed arrows). This is because irreversible loss of tracee occurs not only by direct outflow from the compartment but also from other compartments after interconversion between acids has occurred.
- The sum of the rates of *de novo* incorporation of carbon into each VFA (bold arrows) is equal to the sum of the rates of final disposal of VFA carbon (dashed arrows), but usually differ for each compartment as a result of the VFA interconversions.
- For each VFA, disposal rate should most closely reflect the quantity of VFA available for absorption from the rumen.

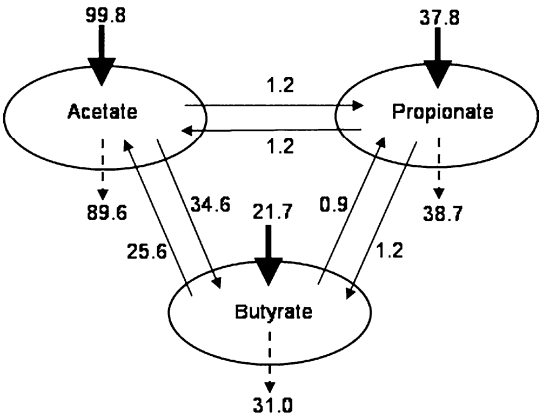

Figure 6. Solution to general three-compartment model of acetate, propionate and butyrate kinetics (g C/d) in the rumen of sheep based on data of Bergman *et al.* (2). Bold arrows = *de novo* synthesis; dashed arrows = final disposal rate

Table 3. Estimates of Methane Production Rate Derived from a Range of Flux Parameters from ^{14}C-VFA Infusions in Sheep. Methane Stoichiometry Explained in Section 3

Model	Flux Parameter	Flux			
		Acet. g C/d	Prop. g C/d	Butyr. g C/d	Methane moles/d
3 pool model	Total flux	129.9	44.5	57.4	3.00
(3 VFA infused)	Net flux	113.8	44.2	50.5	2.59
	De novo	99.8	37.8	21.7	2.04
	Disposal	89.6	38.7	31.0	1.92
	Net effect of including VFA interconversions	–	–	–	0.21
Acetate infusion, SR of acetate determined	Net flux	113.8	47.7	43.5	2.50
Propionate infusion, SR of propionate determined	Net flux	105.4	44.2	40.3	2.31
Acetate infusion, SR of bulk VFA determined	Net flux	92.8	38.9	35.5	2.03

*: Stoichiometry described in section 3

Approach 2. *Infusion of a single ^{14}C-labelled VFA and determination of its unique specific radioactivity*

A single infusion of one ^{14}C-VFA and determination of its plateau SR allows VFA production to be determined and stoichiometric estimates of methane production. The net flux of the VFA (net flux equation; Eqn. 5.) is obtained but no data on recycling is generated. Assuming the molar proportion of the component VFA in rumen fluid reflect their net flux, the net flux of each VFA is derived from the measured flux of the one infused VFA. The data of Bergman *et al.* (2) indicate that if only ^{14}C acetate had been infused, the estimates of acetate, butyrate and propionate net flux would have been 113.8, 47.7 and 43.5 g/d, respectively (Table 3). In contrast, the infusion of ^{14}C-propionate and determination of the SR of propionate would lead to slightly lower estimates of VFA production. This is a consequence of a lesser proportion of propionate carbon recycling into the propionate pool than occurs for acetate, but no measure of recycling can be obtained from any single acid infusion (Table 3).

Approach 3. *Infusion of a single ^{14}C-labelled VFA and determination of the radioactivity in bulked VFA*

A less complex and less expensive procedure that can still give useful information, especially for hydrogen and methane production, was described by Weller *et al.* (34).

A single VFA (e.g. ^{14}C-labelled acetate) is infused and rumen fluid samples are collected after the plateau SR is reached. All VFA are steam distilled simultaneously and the total VFA concentration (mmol acid/mL of distillate) and total radioactivity (DPM/mL of distillate) are determined so that the mean SR of VFA (DPM/mmole VFA) can be calculated. The DPM/mg total VFA-carbon during an intraruminal ^{14}C-acetate infusion was calculated from the experiments of Bergman *et al.* (2) Allowance was made for the proportion of VFA carbon in the total VFA contributed by acetate, propionate and butyrate to give the SR of all three VFA that would have been found in a single distillate (i.e. 16.9 μCi/g C, cf. row 1 of Table 2). The net flux of combined VFA was estimated using the equation $SR_0 = \text{Sum}(A_i)$ (5.40 mol/d) and net fluxes of the individual VFA were then determined by multiplying combined net flux rate by the molar proportions of each acid.

This gave flux rates of 3.87, 1.08 and 0.74 mol/d or 92.8, 38.9 and 35.5 g C/d for acetate, propionate and butyrate, respectively (Table 3). These net flux estimates are fairly similar to the estimates of de novo incorporation for each VFA as determined by separate infusions of all three VFA, with the greatest error being associated with butyrate. This is on account of butyrate having a different absorption: ruminal concentration relationship to other VFA and because of the large amount of butyrate that is produced from acetate. Weller *et al.* (34) have undertaken a similar analysis and reached similar conclusions; however their estimates of net production of combined VFA (mol/d) differed depending on which VFA tracer was used, viz. 5.7 with propionate, 5.4 with acetate and 5.2 with butyrate compared with 5.4 when all three VFA tracers were used.

3. STOICHIOMETRIC ESTIMATION OF METHANE PRODUCTION FROM VFA PRODUCTION DATA

As indicated in the introduction, methane production in the rumen helps to limit hydrogen accumulation in anaerobic rumen contents. This in turn, prevents 'feedback inhibition' of reactions in the fermentation pathways, namely glycolysis, acetate production and butyrate production, which would reduce the rate of organic matter digestion. The hydrogen is removed from reducing agents such as NADH produced within these pathways and the majority is used to reduce CO_2 to methane gas that is then removed from the animal with other expired gases.

If the fermentable organic matter (cellulose, starch etc.) entering the rumen is considered to be degraded via glucose, the pathways of VFA formation can be represented according to the scheme in Figure 7. (The scheme is a simplification because degradation of other organic materials such as proteins is not explicitly represented.)

The individual pathways present in most micro-organisms are depicted in Figure 7, which also summarizes the hydrogen inputs and outputs associated with production of each VFA.

The pathways in Figure 7 show that NADH is produced in glycolysis and also when acetate and butyrate are formed from the resulting pyruvate. NAD^+ is

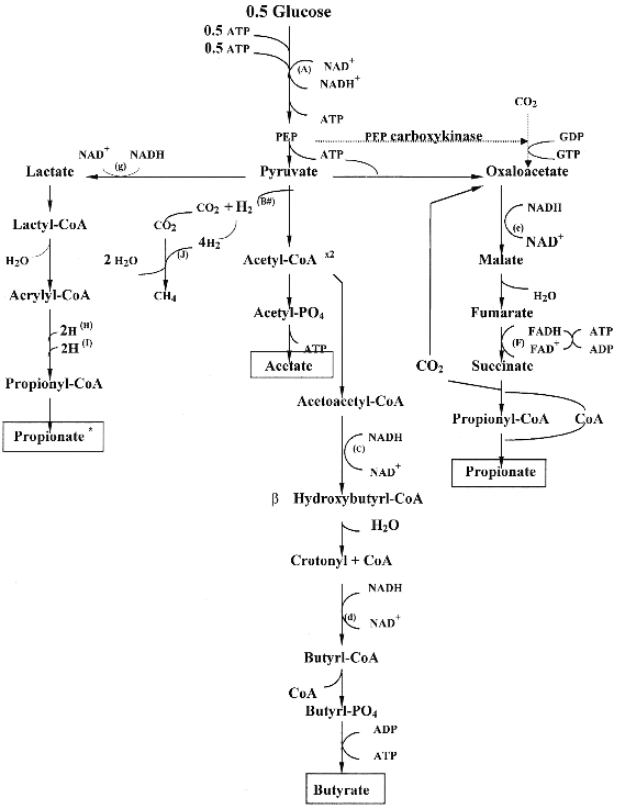

Figure 7. Pathways leading to VFA production in rumen. Hydrogen (2H) utilized and consumed in the synthesis of acetate, propionate and butyrate are summarized. After Gottschalk (10)

regenerated when propionate (and valerate – not shown in Fig. 7) are formed, or when the NADH dissociates to form H_2 gas. High rates of fermentation of feed carbohydrates result in increases in intracellular NADH concentrations that may tend to increase further if microbial growth is impaired for any reason, and fermentation and growth are uncoupled. NADH is in reversible equilibrium with H_2 gas, represented here as 2(H) in the rumen.

$$\text{NADH} + \text{H}^+ \leftrightarrow \text{NAD}^+ + 2(\text{H}) \quad (6)$$

Regeneration of NAD^+, promoted by the removal of 2(H), is necessary to enable fermentation reactions that generate NADH (glycolysis, and acetate and butyrate

formation) to continue. Thus, synthesis of acetate and propionate are complementary processes and often occur simultaneously in anaerobic microbes.

When NADH concentrations increase, the normally low rumen H_2 concentration (about 10^{-4} atmospheres; Wolin (36)) moves the equilibrium to the right, promoting release of more gaseous H_2. (H_2 is also released from formate derived from pyruvate by some methanogenic species.) Gaseous H_2 seldom accumulates in the rumen because it is apparently transferred from hydrogen-producing microbes to methanogenic organisms that obtain ATP by using it to reduce CO_2 to CH_4, according to the following equation

$$(7) \qquad CO_2 + 8(H) \quad \rightarrow \quad CH_4 + 2H_2O$$

If all 2(H) produced during the production of these VFA ('A' is acetate etc) were used in methane synthesis, then potential methane production would be given by

$$(8) \qquad 0.5A + 0.5B - 0.25P - 0.25V$$

Predicting methane production from the above equation would be valid only if:

- these VFA were the only fermentation end-products (i.e. no hydrogen is used in cell polymer production)
- no free H_2 escaped from the rumen
- the microbial digestion processes were strictly anaerobic (i.e. no H_2 is used to reduce O_2 to H_2O)
- H_2 is not used in other reactions, e.g. the reduction of sulphates to sulphides or of double bonds in fatty acids.

In real life, production of methane will be lower than the equation predicts because these assumptions are not totally correct. We know, for example, that some NADH, or 2(H), is oxidized to provide energy for synthesis of cell polymers (e.g. lipids, amino acids and nucleic acids) during growth of cells, and in various other redox reactions (6).

In summary, if the net disposal rates of individual VFA are known or can be estimated, the net NADH and hydrogen generation can calculated and methane production predicted. If ATP production during hexose fermentation is also calculated, estimates can be made of potential cell synthesis and of the 2(H) removed by this synthesis. This will allow a better prediction of methane production.

One means of making the relevant calculations is to use a model similar to that proposed by Czerkawski (6). This model has been implemented as software by Nolan (24).

At this point it is also worth noting that many workers have relied on molar concentrations of the VFA, expressed as molar percentages, to develop equations for predicting methane production, rather than molar *de novo* synthesis rates. This introduces another error that is often not explicitly stated. This error will arise if it is assumed that molar concentrations of individual VFA (expressed as molar percentages of total VFA) will correctly represent individual VFA disposal rates. Even though quite good relationships between VFA concentrations in rumen fluid and net production rates have been demonstrated (19, 15, 33), interconversions of

VFA are not fully accounted for, and factors such as changes in rumen pH (11, 28) and osmotic pressure (21) alter these relationships to some extent and will affect some VFA more than others (7).

3.1. General Discussion

Since VFA are the principal energy source for ruminant tissues, the techniques of measuring VFA production have made major contributions to understanding of ruminant physiology and energetics. There are however, a number of possible experimental procedures, and a number of descriptions of 'VFA production' that can be derived from any one *in vivo* procedure used to measure VFA flux. Selecting the appropriate measure of VFA flux to use in taking a stoichiometric approach to predicting methane production is somewhat subjective and it is easy to have circular arguments about which flux should be used.

The comparison summarized in Table 3 identifies that the differences in estimated methane production arising from use of different VFA flux measures may be up to 25% and this variation due to flux measurement will magnify the already considerable experimental errors associated with making ruminal measurements over 6 h to 3 d. Using the data from studies by Bergman *et al.* (2), Leng and Leonard (18) and Leng and Brett (19), Rowe (27) attempted to calculate errors associated with the procedures mentioned above and concluded that (a) combined production rate of acetate, propionate and butyrate was always overestimated by the single VFA infusion technique (3.5% $\pm$ SE 1.1, n=6), (b) accuracy of estimates of 'effective production rate' of individual VFA was variable, the difference between predicted values and those estimated by using individual infusions of each VFA ranging from -36% to $+44\%$ of the mean, which is more than our estimates in Table 3.

On the basis of modelling conducted to create Table 3, it can be recommended that for most (grain and roughage) diets, the single acid infusion procedure (preferably using propionate) should be used, with isolation of that acid from the VFA and determination of its SR. This approach is intermediate in terms of complexity and also in its ability to avoid calculating the interconversion of the VFA. Consequently, the estimation of daily methane production by this method (2.49 moles/d) was 89% of that estimated if the net flux of each VFA had been measured separately, but 14% higher than if the SR of bulked VFA had been relied on. The error in estimating methane production from ignoring the interconversion of VFA (acetate : butyrate, butyrate : propionate) of 0.21 moles was less than 10% of methane production.

On diets that have high proportions of butyrate (such as molasses based diets), this procedure can be expected to generate greater error. This is because acetate: butyate interconversion and because the relationship between butyrate proportion and absorption differs from that of acetate and propionate (7). Only a 'three-acid' infusion or injection protocol will suffice for such diets.

From *in vitro* studies, where rises in concentration of VFA and methane are subject to far less experimental error than occurs in whole animal studies, stoichiometry does typically produce good hydrogen recoveries although again, recoveries fall when

methanogenesis is interrupted. *In vivo*, however, stoichiometry should not be viewed as an accurate way of estimating methane production. Clearly, the most appropriate way to determine methane production in a ruminant is to measure the amount produced by direct methods (as described in other chapters of this IAEA text) rather than by using predictions based on stoichiometric relationships.

If the animal is housed in a chamber or an enclosed room the mixed gases released (both expired and flatus) can be analysed for methane concentration. If the rates of flow of gases from the chamber or room are determined, then a direct estimate of methane release from all segments of the gut of the animal can be determined directly. Estimates can also be made in enclosed chambers by determining the small increase in methane concentration over short periods.

Probably the next most appropriate means of determining methane production is to estimate it by tracer dilution methods. Methane labelled with ^{14}C, ^{3}H or deuterium can be used and the SR of methane determined over time (14). The precision of these estimates will depend on how effectively the tracer and tracee are mixed in the rumen, and on the analytical techniques. The method based on constant release of SF_6 in the rumen is another potential method, and the possible errors have been discussed (32, 4, 9).

Finally, VFA production can be determined *in vivo* as described above and predictions of methane production can be made using stoichiometric equations. This is probably the least appropriate measurement option because the predictions based on VFA production will theoretically result in an overestimation of the true methane production due to assimilation of hydrogen during the synthesis of microbial polymers and in other reactions are not been allowed for. The importance of these unspecified reactions is difficult to gauge and may depend on the mix of species of bacteria and other microbes present. Their effects may be greater when inhibitors of methane production have been included in the animal's diet.

While determinations of ruminal VFA production *in vivo* are helpful for digestive and energetic studies of diet utilization and the animal, they should not be looked upon as the most appropriate method to quantify enteric methane production. Leng (7) envisaged measurement of methane production as a simple way of estimating VFA production rather than the reverse. This is because (a) the current requirement for cannulated animals, (b) the complex laboratory procedures associated with isolating individual VFA and (c) the errors associated with assuming rumen conditions are constant over the 3–4 d of experimentation. Consequently, for laboratories seeking a single direct means of measuring only enteric methane production, procedures utilizing direct methane tracers or respiratory hoods or chambers are recommended.

REFERENCES

1. Binnerts W.T., A.T. Van Klooster, and A.M. Frens. 1968. Soluble chromium indicator measured by atomic absorption in digestive experiments. Vet. Rec. **82**: 470.
2. Bergman, E.N., R.S. Reid, M.G. Murray, J.M. Brockway, and F.G. Whitelaw. 1965. Interconversions and production of volatile fatty acids in the sheep rumen. Biochem. J. **97**: 5–58.

3. Boadi, D.A., K.M. Wittenberg, and A.D. Kennedy. 2002. Validation of the sulphur hexafluoride (SF6) tracer gas technique for measurement of methane and carbon dioxide production by cattle. Can. J. Anim. Sci. **82**:125–131.
4. Blake, J.S., D.N. Salter, and R.H. Smith. 1983. Incorporation of nitrogen into rumen bacterial fractions of steers given protein-and urea-containing diets. Ammonia assimilation into intracellular bacterial amino acids. Br. J. Nutr. **50**:769–782.
5. Czerkawski, J.W. 1986. An introduction to rumen studies. Pergamon Press, Oxford.
6. Czerkawski. J.W. and G. Breckenridge. 1975. Separation and determination of mass and radioactivity of fermentation gases. Anal. Biochem. **67**:476–484.
7. Danielli, J.F., M.W.S. Hitchcock, R.A. Marshall, and A.T. Phillipson. 1945. The mechancism of absorption from the rumen as exemplified by the behaviour of acetic, propionic and butyric acids. J. Expl. Biol. **22**:75–84.
8. Demeyer, D.I. and C.J. Van Nevel. 1975. Methanogenesis, an integrated part of carbohydrate fermentation, and its control. In. I.W. McDonald and A.C.I. Warner (eds.), *Digestion and metabolism in the Ruminant*, p 366–383. (Refer p.12 Ref. 6) The University of New England Publishing Unit, Armidale, Australia.
9. Fredeen, A.H., S. Cooper, Tate, M. Main, and J. Yuill. 2004. Comparison of whole body breath-sampling methods of measuring methane production in dairy cows. Proc. Canadian Soc. Anim. Sci. Annual Meeting July 2004. RP18, p41.
10. Gottschalk, G. 1986. Bacterial metabolism (2^{nd} ed) Springer-Verlag, New York Inc.
11. Gray, F.V. 1948. The absorption of volatile fatty acids from the rumen II. The influence of pH on absorption. J. Exp. Biol. **25**: 135–144.
12. Gray, F.V., G.B. Jones, and A.F. Pilgrim. 1960. The rates of production of volatile fatty acids in the rumen. Aust. J. Agric. Res. **11**:384–388.
13. Hegarty, R.S., J.V. Nolan, and R.A. Leng. 1991. Sulphur availability and microbial fermentation in the fauna-free rumen. Arch. Anim. Nutr. **41**:725–736.
14. Hegarty, R.S, R.A. Leng, and J.V. Nolan. 2005. Measurement of methane production rate in the rumen using isotopic tracers, pp 93–103.
15. Leng, R.A. 1970. Formation and production of volatile fatty acids in the rumen. In. A.T. Phillipson (ed.), *Physiology of digestion and metabolism in the ruminant*, p 406–421. Oriel press ltd., Newcastle upon Tyne.
16. Leng, R.A. 1971. Measurement of fermentation rate in the rumen. Proceedings: International Symposium on Use of Isotopes and Radiation in Agriculture and Animal Health International Atomic Energy Agency, New Delhi. pp 745–757.
17. Leng, R.A. and R. Leonard. 1965. Measurement of the rate of production of acetic, propionic and butyric acids in the rumen of sheep Brit. J. Nut. **19**:469–484.
18. Leng, R.A. and R. Leonard. (1965). Loss of methyl tritium from [^{3}H]acetate in rumen fluid. Nature **207**:760–761.
19. Leng, R.A. and D.J. Brett. 1966. Simultaneous measurements of the rates of production of acetic, propionic and butyric acids in the rumen of sheep on different diets and the correlation between production rates and concentrations of these acids in the rumen. Br. J. Nutr. **20**:541–552.
20. Leng, R.A., J.L. Corbett, and D.J. Brett. 1968. Rates of production of volatile fatty acids in the rumen of grazing sheep and their relation to ruminal concentrations. Br. J. Nutr. **22**:57–68.
21. Lopez, S., F.D. Hovell, and N.A. MacLeod. 1994. Osmotic pressure, water kinetics and volatile fatty acid absorption in the rumen of sheep sustained by intragastric infusions Br. J. Nutr. **71**:153–168.
22. Nolan, J.V. and R.A. Leng. 1974. Isotope techniques for studying the dynamics of nitrogen metabolism in ruminants. Proc. Nutr. Soc. **33**:1–8.
23. Nolan, J.V., B.W. Norton, and R.A. Leng. 1976. Further studies of the dynamics of nitrogen metabolism in sheep. Br. J. Nutr. **35**:127–147.
24. Nolan, J.V. 1998. Stoichiometry of rumen fermentation and gas production. In. P.J. Reyenga and S.M. Howden (eds.), *Meeting the Kyoto Target. Implications for the Australian Livestock Industries*, p 21–28, Bureau of Resource Sciences. Canberra.

25. Oldham, J.D., I. Bruckental, and A. Nissenbaum. 1980. Observations on rumen ammonia metabolism in lactating dairy cows. J. Agric. Sci., Camb. **95**: 235–238.
26. Orskov, E.R., W.P. Flatt, and P.W. Moe. 1968. Fermentation balance approach to estimate extent of fermentation and efficiency of volatile fatty acid formation in ruminants. J. Dairy Sci. **51**:1429–1435
27. Rowe, J.B. 1978. Studies on the interrelationship between fermentation, digestion and metabolism in sheep. PhD Thesis. The University of New England, Armidale NSW.
28. Russell, J.B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vivo. J. Dairy Sci. **81**:3222–3230.
29. Sheppard, A.J., R.M. Forbes, and B. Conner Johnson. 1959. Rate of acetic acid production in the rumne determined by isotope dilution. Proc. Soc. Expl. Biol. **101**:715–717.
30. Sutherland, T.M., W.C. Ellis, R.S. Reid, and M.G. Murray. 1962. A method of circulating and sampling rumen contents of sheep fed on ground pelleted foods. Br. J. Nutr. **16**:603–614.
31. Srinivas B. and B.N. Gupta. 1997. Rumen fermentation, bacterial and total volatile fatty acid (TVFA) production rates in cattle fed on urea-molasses-mineral block lick supplements. Anim. Feed Sci. Tech. **65**:275–286.
32. Ulyatt, M.J., B.N. Baker, G.J. McCrabb, and K.R. Lassey. 1999. Accuracy of SF_6 Tracer Technology and Alternatives for Field Measurements. Aust. J. Agric. Res. **50**:1329–1334.
33. Van de Walt, J.G. and B.J. Breil. 1976. Volatile fatty acid metabolism in sheep. 1. Average daily volatile fatty acid production in the rumen of sheep fed lucerne hay. Onderstepoort J. Vet. Res. **43**:11–21.
34. Weller R.A., F.V. Gray, A.F. Pilgrim, and G.B. Jones. 1967 The rates of production of fatty acids in the rumen. IV. Individual and total fatty acids. Aust. J. Agric. Res. **18**:107–118.
35. Weston, R.H. and J.P. Hogan. 1968. The digestion of pasture plants by sheep. 1. Ruminal production of volatile fatty acids by sheep offered diets of ryegrass and forage oats. Aust. J. Agric. Res. **19**:419–432.
36. Wolin, M.J. 1975. Interactions Between the Bacterial Species of the Rumen. In. I.W. McDonald and A.C.I. Warner (eds.), *Digestion and Metabolism in the Ruminant*, p. 134–148, University of New England Publishing Unit, Armidale NSW Australia.