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Methane production in the rumen and lower gut of sheep given lucerne chaff: effect of level of intake

BY R. M. MURRAY,* A. M. BRYANT† AND R. A. LENG

Department of Biochemistry and Nutrition, Faculty of Rural Science, University of New England, Armidale, New South Wales, 2351, Australia

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1. Methane production rates were estimated simultaneously in the rumen and caecum of sheep given 200, 400, 600, 800 and 1000 g lucerne (*Medicago sativa*) chaff/d using isotope dilution techniques. Estimates were also made of volatile fatty acid (VFA) production in the rumen at each level of feeding. In all studies three to four animals were used at each level of intake.

2. Production of VFA and of methane were both related to digestible energy (DE) intake. Regression lines for both VFA production and methane production *v.* DE intake had significant intercepts indicating an input of endogenous, fermentable organic matter into the rumen in excess of 50 g/d.

3. The values obtained for rates of methane production were compared with those calculated from stoicheiometric equations relating rates of methane and VFA production. Comparisons of methane production with that predicted from DE intake were also made.

4. Balances for digestion of food determined for the rumen indicated that the energies in the end-products were more than 100 % of the DE intakes at low intakes of lucerne chaff. Correction for fermentation of apparent endogenous materials resulted in more realistic values. Endogenous materials appeared to make a significant contribution to VFA and methane production, particularly at low levels of intake.

In an earlier paper (Murray, Bryant & Leng, 1976) a radioactive tracer method was described for the simultaneous measurement of the rates of methane production in the rumen and caecum of sheep. It was suggested that this technique might have possibilities for assessing rumen fermentation rate as an alternative to its estimation based on measurement of the production of volatile fatty acids (VFA). The studies described here were undertaken as part of a programme to study the technique for measuring methane production as a means of estimating the rate of fermentation in the rumen. Therefore measurements of the rates of production of VFA and methane in the rumen were made to allow comparisons between the two techniques in the same animal, and to assist in developing a relationship between VFA production and methane production.

EXPERIMENTAL

Animals and their management

Six adult Merino ewes, each with a permanent rumen fistula, weighing between 38 and 42 kg were used. They were housed indoors in metabolism cages and given one of the following rations: 200, 400, 600, 800 and 1000 g lucerne (*Medicago sativa*) chaff/d and were given one-twenty-fourth of their ration at hourly intervals from automatic feeders. At least three sheep were used at each level of intake and the periods during which each ration was given were randomized and were of at least 4 weeks duration. Throughout the final week, apparent digestibility of the ration was estimated while measurements of rates of methane and VFA production were made on days 6 and 7 of this week, respectively.

* Present address: Department of Tropical Veterinary Science, James Cook University, Townsville, Queensland, 4811, Australia.

† Present address: Ruakura Agricultural Research Station, Hamilton, New Zealand.

Table 1. *Composition (g/kg dry matter* (DM)) *and apparent digestibility of* DM, *organic matter, nitrogen and energy of lucerne* (Medicago sativa) *chaff fed to sheep*

	DM	Organic matter	N	Energy (MJ/kg DM)
Composition	860*	920	38	19·2
Digestibility				
Mean	0·63	0·65	0·76	0·63
SE	0·004	0·004	0·004	0·004

* Air-dried lucerne.

Experimental procedures

Methane production. Total methane production rate and its partition between the rumen and hind gut, were measured as previously described (Murray *et al.* 1976).

Total methane excretion through the mouth was estimated by (1) drawing a known volume of air through the mask per min and measuring the methane in a portion and (2) by measuring the specific radioactivity in the same portion and relating this to the infusion rate into the rumen of radioactivity in methane (see Murray *et al.* 1976).

VFA production rate. A [U-^{14}C]acetate solution (approximately 10 μCi/ml, 1 μmol/ml) was infused into the rumen at a rate of 1·2 ml/min. Sampling of rumen contents began 4 h after the start of infusion. Rumen fluid (5 ml) was collected every 10 min for 1 h and bulked to give one 30 ml sample. Four bulked samples were collected in a 4 h period. Specific radioactivity (SR) content for each VFA in each bulked sample was determined as described by Leng & Leonard (1965). The weighted SR (μCi/mmol) of each VFA calculated from their molar proportions, were summed to give the total VFA SR which was divided into the rate of infusion (μCi/min) to calculate total VFA production rate (mmol/min). Acetate, propionate and butyrate (plus higher and branched chain VFA) production rates were calculated from the total VFA production rate by assuming that these were produced in proportion to their concentration (see Leng, 1970).

Statistical analysis. Changes in measurements of fermentation rate with changes in intake were analysed by regression analysis. Analyses of variance were used to compare values between sheep and experiments.

RESULTS

Digestibility

The proximate analysis of the lucerne chaff used throughout the experiment is shown in Table 1. The apparent digestibility of dry matter, organic matter, energy and nitrogen did not vary significantly with level of intake, and the mean coefficients for each component are shown in Table 1.

Comparison of methane excretion and methane estimated in expired air by isotope dilution

In five sheep given 800 g lucerne chaff/d methane production was estimated over 1 h periods from a knowledge of air flow through the mask and the content of methane in that air, or from the SR of methane collected from the mask at the same time. Several sampling periods (between three and five) for each sheep were averaged. Methane production was not significantly different when estimated by the two methods and was (mean $\pm$ SE) 20·1 $\pm$ 1·9 (5) and 19·8 $\pm$ 0·9 (5) ml/min respectively by the total collection and isotope methods respectively.

Table 2. *Variation in plateau specific radioactivity during the period used to estimate production rates of volatile fatty acids (VFA) and methane in the rumen of sheep given lucerne* (Medicago sativa) *chaff and intraruminal infusions of [U-^{14}C]acetate and [^{3}H]methane*

(Mean values for six determinations plus the grand means, their standard error (SE) and coefficient of variation (CV). Infusion rate was 20 μCi [U-^{14}C]acetate or 1·6 μCi [^{3}H]methane in 1·2 ml/min)

Period after start of infusion (h)	Plateau specific radioactivity: VFA (μCi/μmol)	Plateau specific radioactivity: methane (μCi/ml)
5	8·0	154
6	7·2	161
7	8·0	163
8	8·6	154
Mean	7·9	158
SE	0·3	2·7
CV	3·8	1·7

Variations in plateau SR of methane and VFA during separate infusions of [^{3}H]methane and ^{14}C-labelled VFA respectively

The percentage variation in estimates of VFA production rates were more than twice that for estimates of methane production rates. Details of these experiments are shown in Table 2.

Methane production. The relationship between digestible organic matter (DOM) intake (X, g/d) and total methane production (Y_T, l/d), methane production within the rumen (Y_R, l/d), and within the hind gut (Y_H, l/d) were found to be respectively:

$$Y_T = 2{\cdot}81 + 0{\cdot}042\ (\pm 0{\cdot}003)X \quad \text{(RCV 10·7)}, \tag{1}$$

$$Y_R = 2{\cdot}89 + 0{\cdot}036\ (\pm 0{\cdot}003)X \quad \text{(RCV 12·6)}, \tag{2}$$

$$Y_H = -0{\cdot}08 + 0{\cdot}006\ (\pm 0{\cdot}001)X \quad \text{(RCV 28·9)}, \tag{3}$$

where RCV is residual coefficient of variation. The relationships between digestible energy (DE) intake and methane production in the rumen and hind gut are shown in Fig. 1. The mean ($\pm$ SE) amount of methane produced in the rumen, which was 88·7 $\pm$ 3·7% of the total methane production, tended to decrease as intake increased, but this decrease was not statistically significant. The mean ($\pm$ SE) amount of methane excreted via the anus was 13·4 $\pm$ 7·5% of methane produced in the hind gut and although this value tended to increase with intake, the increase was not significant. However, rumen methane production (mol/kg DOM) decreased significantly ($P < 0{\cdot}01$) as DOM intake (X, g/d) was increased; i.e.

$$Y_R = 2{\cdot}78 - 0{\cdot}002\ (\pm 0{\cdot}0009)X \quad \text{(RCV 23·4)}. \tag{4}$$

VFA production. Fig. 1 illustrates the relationship between rate of VFA production within the rumen and DE intake. The molar proportion of individual VFA in the rumen fluid of the sheep did not change with level of feeding, being 700, 190, 60 and 50 mmol/mol VFA respectively for acetic, propionic, butyric and the branched- and higher-chain acids. However, the total concentration of the acids increased significantly ($P < 0{\cdot}01$) as intake was increased, as did the total quantity of VFA produced. The decrease in VFA production (per kg DOM) with increasing intake was not statistically significant.

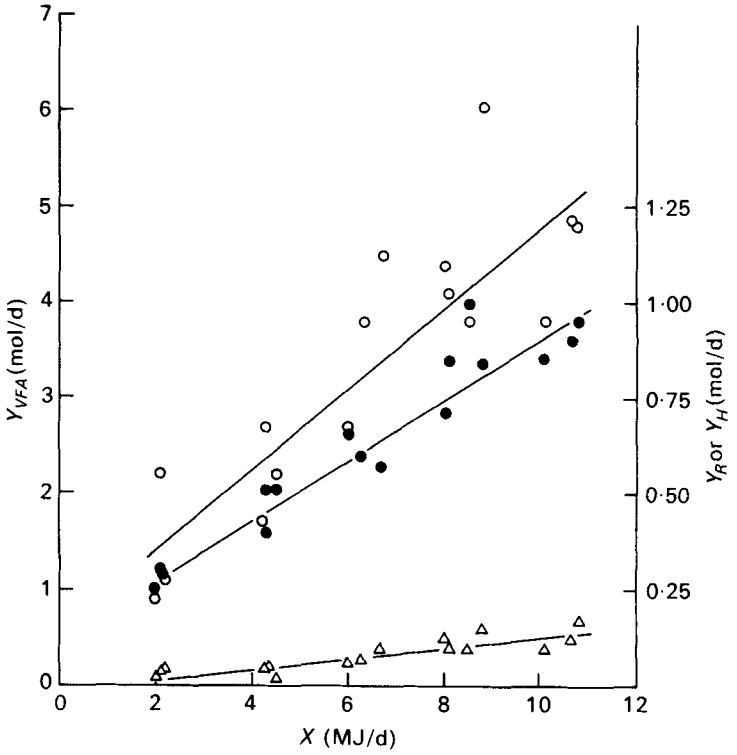

Fig. 1. Relationships between digestible energy intake (X; MJ/d) and (O) volatile fatty acid production (mol/d; Y_{VFA}) and (●) methane production (mol/d) in the rumen (Y_R) and (△) methane production in the hind gut (Y_H) of sheep given lucerne (*Medicago sativa*) chaff and intraruminal infusions of [U-^{14}C]acetate and [^{3}H]methane.

$Y_{VFA} = 0{\cdot}600 + 0{\cdot}423\ (\pm 0{\cdot}065)\ X$ (RCV 22·6),

$Y_R = 0{\cdot}129 + 0{\cdot}078\ (\pm 0{\cdot}007)\ X$ (RCV 12·5),

$Y_H = -0{\cdot}003 + 0{\cdot}013\ (\pm 0{\cdot}002)\ X$ (RCV 29·5),

where RCV is residual coefficient of variation.

Relationship between the measured methane production and that calculated from VFA production using stoicheiometric principles

The theoretical rumen methane production was calculated using balance equations from the relationship between VFA production and methane production according to Hungate (1968) and Leng (1970) (Table 2).

The regression equations of predicted methane production *v.* measured methane production in the rumen were:

$$Y_{(Leng)} = 0{\cdot}05 + 1{\cdot}08\ (\pm 0{\cdot}202)X \quad \text{(RCV 26·4)}, \tag{5}$$

$$Y_{(Hungate)} = 0{\cdot}06 + 1{\cdot}24\ (\pm 0{\cdot}248)X \quad \text{(RCV 28·2)}, \tag{6}$$

where $Y_{(Leng)}$ is methane production (mol/d) according to Leng (1970), $Y_{(Hungate)}$ is methane production (mol/d) according to Hungate (1968), X is measured methane production (mol/d).

The intercepts of the regression lines were significantly different from zero. The regression coefficient of the equation derived from the stoicheiometry preferred by Hungate (1968)

Table 3. *Comparison between mean values for measured and calculated methane production for sheep given lucerne* (Medicago sativa) *chaff and intraruminal infusions of* [*U*-^{14}*C*]*acetate and* [^{3}*H*]*methane*

(Total methane was calculated from gross energy intake using the equation of Blaxter & Clapperton (1965) (*a*) and rumen methane production was calculated from VFA production using the stoicheiometric relationships of Leng (1970) (*b*) and Hungate (1968) (*c*); for details, see below)

Level of feeding air-dried lucerne chaff (g/d) ...	200	400	600	800	1000
No. of measurements	3	3	3	4	3
Total methane production (mol/d):					
Measured	0·31	0·52	0·69	0·95	1·03
Calculated (*a*)	0·31	0·62	0·88	1·14	1·39
Rumen methane production (mol/d):					
Measured	0·28	0·48	0·61	0·83	0·90
Calculated (*b*)	0·31	0·47	0·79	1·00	0·98
Calculated (*c*)	0·36	0·55	0·90	1·16	1·12

was significantly ($P < 0{\cdot}01$) greater than 1, but that of the regression coefficient of the equation derived from Leng's (1970) stoicheiometry was not significantly ($P > 0{\cdot}05$) different from 1. The stoicheiometry used by Hungate (1968) over-estimated actual methane production by 32%. The individual results are shown in Table 3.

Production of methane in these sheep was over-estimated by 10–30%, using the regression equation of gross energy (GE) intake *v.* total methane production suggested by Blaxter & Clapperton (1965) (Table 3). The relationship of predicted value *v.* measured value was:

$$Y_{(\text{Blaxter \& Clapperton})} = -0{\cdot}06 + 1{\cdot}32\ (\pm 0{\cdot}093)X \quad (\text{RCV } 11{\cdot}6), \tag{7}$$

where $Y_{(\text{Blaxter \& Clapperton})}$ is predicted from the equation $C_m = 1{\cdot}30 + 0{\cdot}112D + L(2{\cdot}37 - 0{\cdot}05D)$ (Blaxter & Clapperton, 1965), where C_m is methane energy (kcal/100 kcal GE), D is digestibility of GE, L is the level of intake relative to maintenance.

Relationship between rumen VFA and methane production

The molar ratio, VFA production: methane production did not change significantly for the range of intakes studied, being $4{\cdot}86 \pm 0{\cdot}85$ mol VFA/mol methane. Similarly the molar ratio, acetate $+ 2 \times$ butyrate: methane did not vary significantly from $4{\cdot}31 \pm 0{\cdot}65$ (mean $\pm$ SE) (see Leng, 1970).

Calculated rumen fermentation balance results

Balance results calculated from measured rumen fermentation products using the equations of Baldwin, Lucas & Cabrera (1970) and Leng (1970) are shown in Table 4, together with the predicted contribution of rumen fermentation to the apparent DE.

DISCUSSION

Comparison of total collection and isotope dilution methods for measuring methane production by sheep have indicated that the isotope technique gives an accurate estimate.

The results presented in this paper suggested that the measurement of methane production by sheep using the isotope dilution technique may be a more precise method of assessing fermentation rate in the rumen than the measurement of VFA production. The precision with which the plateau SR of methane could be measured suggests that the technique has overcome the apparent problems of mixing of isotopes in the liquid pool in the rumen (see Leng, 1970). The variation (%) in estimates of VFA production rates, although more than

twice that for methane production rate estimates must be considered small however. Undoubtedly this is because errors associated with mixing of labelled VFA within the rumen (infusion rate 1·2 ml/min) were reduced as far as was practicable, and representative samples were obtained from the VFA pool (sampling every 10 min). However, despite this lack of variation in plateau SR, the relationship between VFA production and DE intake compared with that of methane production and DE intake (Fig. 1) was relatively variable, indicating that DE intake may be more accurately predicted from measurements of methane production as compared to that from VFA production.

Wolin (1960) and Hungate (1960, 1966) suggested a stoicheiometric relationship between carbohydrate fermented and the various end-products of this fermentation. This was later modified by Hungate (1968) who suggested that to balance the stoicheiometry the hydrogen used in cell synthesis must be considered. Leng (1970) suggested that all the electrons generated as reduced co-enzymes may not be converted to hydrogen for conversion to methane and proposed a further stoicheiometry.

In this stoicheiometry since about one-third of the electrons are generated as NADH and two-thirds as hydrogen gas, the recovery of hydrogen in methane from carbohydrate fermented will be about 60–66% (see Leng, 1970; Leng, 1974). Implicit in this assumption is that the majority of the electrons not accounted for in methane are incorporated into microbial cells (i.e. the reduced co-enzymes are oxidized in the synthesis of microbial cells) or are accepted by miscellaneous H_2 acceptors such as nitrate, sulphate, unsaturated fatty acids etc. (see Demeyer & Van Nevel, 1975). In general, it has been suggested that electrons from NADH are not incorporated extensively into microbial cells since the microbes are not much more reduced than the carbohydrate fermented (Demeyer & Van Nevel, 1975). However, this suggestion assumes that the starting material for cell synthesis is the available carbohydrate. One mechanism by which electrons could be incorporated into microbial cells without increasing their hydrogen content is that involving CO_2-fixation. For instance, the pathway by which propionate is produced from carbohydrates involves CO_2 fixation by pyruvate to oxalacetate. This is then converted via the dicarboxylic acids, malic, fumaric and succinic acids to propionic acid (see Leng, 1974). If these dicarboxylic acids enter synthetic reactions such as the formation of aspartate (from oxaloacetate) or glutamate (from succinate via α-ketoglutarate), cellular synthesis must involve CO_2 fixation and allows the micro-organisms themselves to act as an electron sink. There are possibly other mechanisms by which CO_2 fixation occurs before incorporation of substrates into microbial cells. The synthesis of microbial dry matter might therefore be better represented by the following equation: (cf. Demeyer & Van Nevel, 1975)

$$C_6H_{12}O_6 + 1{\cdot}2\ NH_3 + n\,(2H) + mCO_2 \rightarrow C_6H_{9{\cdot}85}O_{2{\cdot}99}N_{1{\cdot}20} + 3{\cdot}01\ H_2O$$

where n and m are unknown at the present time.

The relationships proposed by Leng (1970) and Hungate (1968) were used to calculate expected methane production from VFA production and the results indicate that the relationship proposed by Leng (1970) more closely describes the relationship between VFA and methane in the rumen of these sheep on this diet. Demeyer, Henderickx & Van Nevel (1972) have also prepared a fermentation balance corrected for the hydrogen used in anabolism of rumen microbes; however its use requires previous knowledge of the quantity of carbohydrate digested in the rumen. From the calculated values, it has been possible to build up a balance sheet for substrate digestion within the rumen (Table 4). Heat of fermentation was calculated by subtracting the energy value of the VFA and methane produced from that of the hexose apparently fermented. Estimates of ATP availability to the microbes have also been made using the stoicheiometric equations of Leng (1970) and from these estimates, the quantities of microbial cells produced have been calculated, assuming that approxi-

Table 4. *Balance results calculated from rumen fermentation products and predicted contribution of rumen fermentation to the apparent digestible energy* (DE) *at different levels of intake of lucerne* (Medicago sativa) *chaff for sheep*

(Mean values for sixteen determinations using six sheep; four determinations were made at the 800 g/d level of feeding and three determinations were made at each of the other levels of feeding)

Level of feeding (g/d)	Microbes* (g)	VFA (mol)			Methane† (mol)	Heat‡ (MJ)	Estimated energy contribution (% DE)		
		Acetic†	Propionic†	Butyric† and higher acids			VFA	Microbes	Methane + heat
0	0								
200	53	1·05	0·25	0·09	0·28	0·31	72	53	27
400	84	1·62	0·43	0·14	0·48	0·47	55	40	21
600	100	2·68	0·73	0·24	0·61	0·79	62	46	21
800	177	3·40	0·88	0·31	0·83	1·00	60	44	21
1000	172	3·27	0·86	0·32	0·90	0·92	46	34	16

VFA, volatile fatty acids.

* Calculated assuming 16·4 g/mol ATP, and 21 kJ/g dry cells (Baldwin, Lucas & Cabrera, 1970). ATP available to the microbes was calculated from VFA production rates using the stoicheiometric relationships proposed by Leng (1970).

† Measured products.

‡ Calculated by difference (hexose fermented – (VFA + methane)) from stoicheiometric relationships (Leng, 1970).

mately 16·4 g dry cells are produced per mol ATP with an energy value of 21 kJ/g (Baldwin *et al.* 1970). Table 4 shows the quantities of fermentation products produced in the rumen and the proportion of apparent DE appearing as VFA, microbes, methane and heat.

The energy content of all fermentation end products was greater than the DE intake at the lower levels of intake (Table 4) indicating that either there was a considerable input of endogenous materials into the rumen of these sheep or that some of the assumptions are wrong. The increment in methane and VFA production from endogenous sources at any feeding level might be indicated by the intercept of the relationship between these measurements and digestible organic matter or digestible energy intakes which indicates that 0·13 mol methane/d and 0·6 mol VFA/d were produced from endogenous sources which accounts largely for the apparent over-estimation of DE when the products are summed. It also points to a large input of endogenous materials in the rumen which should be considered in models of rumen fermentation. In these animals the endogenous input could be in excess of 50 g DOM/d and may be expected to increase with level of intake.

Implicit in the extrapolation of the linear relationship is that the endogenous inputs into the rumen are constant. Again this might be oversimplified. Although a linear regression might be mathematically the more accurate fit to the data, biologically it may be more rational to fit a quadratic since endogenous inputs should be higher at high intakes (i.e. greater saliva flows, greater abrasion of the rumen epithelium, etc.). Using a linear relationship may thus lead to an overestimation of endogenous inputs at low levels of intake. Possibly increased endogenous inputs at higher food intakes together with decreased fermentation of ingested organic matter and an increased output from the rumen results in the linear relationship found.

The importance of the hind gut in rumen digestion cannot be overlooked, and the fermentation rate in this part of the alimentary tract is about 10% of that in the rumen. Fermentation in the caecum and colon may contribute significantly to digestion under a number of circumstances, for instance in animals on high-grain diets, poor-quality-roughage diets and rations 'protected' against rumen degradation, and sheep on some of these diets are now being studied. As intake of lucerne was increased in sheep, a small but significant ($P < 0{\cdot}01$) decrease was found in the production within the rumen of methane (/kg DOM) demonstrating that post rumen digestion was playing an increasing role with increasing intake. Despite this apparent but small shift in the site of digestion with intake, the pattern of fermentation within the rumen did not alter as demonstrated by the constancy of the ratio, VFA production: methane production and the consistency of the relative proportions of VFA.

It is desirable to be able to estimate quantitatively the contribution of the hind gut to over-all digestion. Until now, workers have relied on sampling from cannulas situated along the alimentary tract using either single cannulas and markers (Hogan & Weston, 1970; Ørskov, Fraser, Mason & Mann, 1970) or re-entrant cannulas and total collection (McRae & Armstrong, 1969; Beever, Coelho da Silva, Prescott & Armstrong, 1972). The use of an isotope dilution technique can give estimates of hind-gut production as well as rumen methane production, and therefore a measure of the relative contribution of rumen and hind-gut fermentation to over-all digestion. That this technique allows the acquisition of such values without surgical interference to the postrumen digestive tract, makes it a useful research tool for the study of ruminant nutrition.

Attempts have been made to predict the losses of energy as methane by sheep and cattle from a knowledge of the amount and type of food they ingest (Swift, Bratzler, James, Tillman & Meek, 1948; Blaxter, 1961). From the results of more than 2500 determinations of the 24 h production of methane by sheep and cattle, Blaxter & Clapperton (1965) have derived equations for predicting methane production from the GE of the diet, the level of

intake and its digestibility. This relationship when used to calculate the expected methane production by the animals in this experiment gave values significantly greater ($P < 0.01$) than the amounts measured. This disparity between calculated and measured methane production was not random, but increased with increasing intake as indicated by the significant difference between the two regression coefficients for each set of values for methane production *v.* DE intake (Table 3). These discrepancies emphasize that more measurements of VFA and methane production rates are required on animals on a variety of diets.

In studies to be reported, where methane production and VFA production were measured over a range of diets, the stoicheiometry of rumen fermentation is variable and lies between those relationships described by Leng (1970) and Hungate (1968).

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