

Measurement of the rates of production of acetic, propionic and butyric acids in the rumen of sheep

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Numerous studies have been made of the quantitative aspects of the production of volatile fatty acids (VFA) in the rumen, and have been reviewed by Rook (1964), Warner (1964) and Annison (1965). Interest in rumen VFA production has recently been intensified because of the findings correlating body-weight increases with the concentration of rumen VFA in lambs on various pastures (Johns, Ulyatt & Glenday, 1963; Rae, Brougham, Glenday & Butler, 1963). Lambs grazing pasture supporting the greatest rate of growth had the greatest concentration of VFA in the rumen and also the highest proportions of propionate and butyrate to acetate. It is still not known, however, whether organic acid levels in the rumen are indicative of production or absorption rates since concentrations of individual acids in the rumen are a balance between production, absorption, passage along the intestinal tract and conversion into other compounds.

Four main groups of experimental techniques have been used to measure production rates of rumen VFA and include: (1) methods based on production of organic acids in rumen fluid incubated *in vitro*; (2) methods based on changes in rumen concentrations after feeding; (3) analyses of the blood draining the rumen either *in vivo* or in isolated preparations; (4) isotope dilution techniques applied either to the rumen or to the whole animal.

Isotope dilution techniques probably offer the most accurate means of measuring production of organic acids in the rumen, since the measurements may be made *in vivo* and with a minimum of disturbance to the animal. Continuous intravenous infusions of [^{14}C]acetate (Annison & Lindsay, 1961), and [^{14}C]propionate and [^{14}C]butyrate (Annison & Lindsay, 1962) have been used to measure the rates at which these compounds enter their body pools. The entry rates for propionate and butyrate have only been measured in experiments in which blood concentrations have been raised by infusion of carrier (Annison & Lindsay, 1962). This was found necessary since only traces of these acids are present in the peripheral blood of sheep (Reid, 1950; McClymont, 1951*a, b*). In addition, the entry rate underestimates production in the rumen because there is considerable metabolism of propionate and butyrate in the rumen epithelium with the production of ketone bodies from butyrate (Pennington, 1952) and of lactate from propionate (Pennington & Sutherland, 1956). With acetate a variable amount of that entering the body pool arises from endogenous sources, especially from fat (see, for example, Annison & White, 1962). For these

reasons measurements of entry rates of VFA in the whole animal are not valid as a means of estimating rumen production or absorption rates.

Isotope dilution methods using single injections of [^{14}C] acids into the rumen have been used to measure the production of VFA (Gray, Jones & Pilgrim, 1960; Sheppard, Forbes & Johnson, 1959), but have the disadvantage that the conversion of one acid into another cannot be estimated. This is a major disadvantage since substantial interconversion of VFA apparently occurs in the rumen (Gray, Pilgrim, Rodda & Weller, 1952; Van Campen & Matrone, 1960; Gray *et al.* 1960; Hungate, Mah & Simesen, 1961; Oppermann, Nelson & Brown, 1961).

In the investigations now described attempts were made to develop a technique for measuring VFA production rates in the rumen *in vivo* using constant infusion of ^{14}C -labelled acids. This technique also allowed estimates to be made of the rates of interconversion of VFA.

EXPERIMENTAL

Animals and feeding regime

Six Merino sheep (aged 5–6 years) with rumen cannulas were housed singly in pens. They were given 75 g lucerne chaff at hourly intervals from 8 am to 7 pm for 5 days. The infusions were made on the last day of each period. Between these periods the sheep were given 900 g lucerne chaff once daily. Animals were allowed water *ad lib.* but it was withheld during infusions.

Digestibility of rations

The digestibility of the ration was determined in all the sheep. They were kept in metabolism cages and given 900 g lucerne chaff once daily for a period of 5 days and then 75 g lucerne chaff at hourly intervals for a further 7 days. Faeces and urine were collected and sampled over the final 3 days. The feed was sampled daily.

Estimations of dry matter, crude protein, ash and fibre were made on the pooled samples of feed and faeces for each sheep (Association of Official Agricultural Chemists, 1960). Gross energies of feed and faeces were estimated by combustion of 1 g amounts of dried materials in a ballistic bomb calorimeter (Miller & Payne, 1959).

Measurements of production rates of VFA

A rubber stopper bored with two holes of $\frac{1}{2}$ in diameter was placed in the mouth of the rumen cannula. A 15 in length of thick-walled Polythene tubing of a $\frac{1}{4}$ in internal diameter was bent in a semicircle and passed through one of the holes in the stopper so that the end rested in the anterior end of the rumen. A second Polythene catheter of $\frac{1}{8}$ in internal diameter was passed through the first tube so that its tip was just in the rumen fluid, and infusions were given through this tube. [$1\text{-}^{14}\text{C}$]acetate, [$1\text{-}^{14}\text{C}$]propionate, and [$1\text{-}^{14}\text{C}$]- or [$2\text{-}^{14}\text{C}$]-butyrate were infused at a rate per min of 1 μmole , 0.5 $\mu\text{C/ml}$, using a micro pump (The Distillers Company Ltd, England). Only one labelled acid was infused at any one time. Rumen samples were obtained

by suction from various sites in the rumen. Four to six samples were taken at any one time and combined. Care was taken to avoid sampling the area surrounding the tip of the catheter.

If blood samples were required a Polythene tube (internal diameter 0.045 in) was inserted into the jugular vein on the evening before the experiment. All animals were fed at hourly intervals before and during infusions which always began at 1.0 pm, when the animal had consumed six of its hourly feeds and rumen VFA were reaching a steady, high concentration. The sheep consumed their ration in 15–20 min and were trained to eat during an experiment. Infusions of a single labelled acid were repeated using different sheep and mean values for individual estimations of the specific activities of acetic, propionic and butyric acids in several experiments on different sheep were calculated.

The pH of the rumen fluid was measured at intervals throughout an infusion, samples being obtained without suction and placed in a beaker under paraffin oil.

Chemical methods

Estimation of total organic acids and percentages of the individual acids in the rumen fluid. Rumen samples of approx. 20 ml were collected in 0.2 ml of 10 N-H₂SO₄ and stored at -10°. For total VFA estimations 1 ml portions of all samples taken between 120 and 240 min after the start of the infusions were pooled and total VFA concentration was estimated by titration, after steam distillation of a 4 ml sample, by the method of Annison (1954). The percentages of the individual acids were measured on the concentrated distillate by gas-liquid chromatography (Erwin, Marco & Emery, 1961) using an Aerograph Hy-Fi Model 600C (Wilkins Instrument and Research, Inc., USA). Acid separations were similar to those of Erwin *et al.* (1961) except that isobutyric acid was clearly separated from propionic acid.

Isolation of acetic, propionic and butyric acids from rumen fluid. After thawing and centrifugation of individual samples, 2 ml of the supernatant fluid were placed in a 100 ml beaker, made alkaline (pH > 8) with N-NaOH and dried under reduced pressure over conc. H₂SO₄. The dried material was mixed with 0.5 ml of 0.25 N-H₂SO₄ and 1 g of silicic acid and then transferred to the top of a silica-gel column prepared by mixing 5 g silicic acid with 2.8 ml of 0.25 N-H₂SO₄ and pouring into a column of 1 cm diameter full of *n*-hexane. Acids were separated with hexane-butanol as eluting solvent (Oppermann, Nelson & Brown, 1957). The mixing device of Donaldson, Tulane & Marshall (1952) was used; the lower vessel contained 125 ml 0.5% (v/v) butanol in hexane and the upper vessel 200 ml 10% (v/v) butanol in hexane.

Fractions of 10 ml were collected off the column. Three peaks were eluted from the silicic acid and these were well separated by several fractions. Butyric acid and branched-chain and higher acids appeared first as one peak, followed by propionic and then acetic acids. The first and last fractions from any one acid peak were always discarded. The acid peaks were collected without titration and placed in 25 ml volumetric flasks and then made up to volume with hexane. Portions (5 ml) were

titrated under CO₂-free conditions with 0.01 N-NaOH in ethanol using bromothymol blue (0.4%, w/v, in ethanol) as indicator.

Fractions isolated by liquid-liquid chromatography were neutralized, dried, and then monitored by gas-liquid chromatography in order to check the efficiency of separation of the acids. Acetic acid was found to be free of any other acids, but the propionic acid peak contained traces (approx. 0.1%) of acetic and butyric acids. These traces were removed by a second-column chromatographic separation. The butyric acid fraction isolated contained, in addition to traces of propionic and acetic acids (approx. 0.1%), isobutyric, isovaleric and valeric acids. The branched-chain and higher acids which constituted 30-40% of the total acid present in the butyric acid peak were found to elute slightly ahead of the butyric and isobutyric acid fractions, and a large proportion could be removed together with the trace contaminations of acetic and propionic acids by two chromatographic separations, the first half of the peak being discarded each time. The proportion of isobutyric acid to other acids in the butyric acid peak was then estimated by gas-liquid chromatography (Erwin *et al.* 1961), and the specific radioactivity of the butyric acid was correspondingly corrected. The validity of this correction was checked by assaying the specific activity of the butyric acid after isolation, using the method of James & Martin (1952) as described below for branched-chain and higher acids.

Isolation of branched-chain and higher acids from rumen fluid. Butyric, valeric, isovaleric and isobutyric acids were isolated as one fraction from other rumen acids by the silica-gel chromatographic procedure. The neutralized sample was dried and the component acids isolated by gas-liquid chromatography, using the method of James & Martin (1952) as modified by Annison (1954). The acids were run off from the collection vessel after they had been titrated. They were freed from indicator by steam distillation at pH 4 and collected in 100 ml water containing a calculated excess of NaOH. The volume of the distillate was reduced to 5-10 ml on a rotary vacuum evaporator at 80° and the sample was finally dried in a 100 ml beaker in a vacuum desiccator over conc. H₂SO₄. Valeric, isovaleric and isobutyric acids were combined and were chromatographed on silica gel using hexane-butanol, and counted as described below. The same procedure was also used to count the butyric acid peak.

Assay of radioactivity of isolated acids. Duplicate 5 ml samples of the combined eluents of the individual acids from the silicic acid column were placed in scintillation vials together with 5 ml of xylene containing 0.4% (w/v) PPO (2-5 diphenyloxazole) and 0.01% (w/v) POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene]. Samples were assayed for radioactivity in a Nuclear Chicago Scintillation System 725 (Nuclear Chicago Corp., Illinois, USA) and were counted with an efficiency of between 50 and 65% using the channel-ratio method of Hendler (1964).

Estimation of the specific activities of carbon dioxide in the rumen and in blood. Rumen fluid or blood samples (5 ml) were added to McCartney bottles containing small tubes of CO₂-free N-NaOH (1 ml). The caps of the containers were tightly closed and 1 ml N-H₂SO₄ was injected into the rumen sample through the cap. After 12 h (for CO₂ absorption) the small tube was removed from the bottle and the NaOH solution was washed into a test tube containing 1 ml (w/v) NH₄Cl. The carbonate was pre-

precipitated as BaCO_3 on adding 0.4 ml 20% (w/v) $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ and plated as previously described (Annison & White, 1961). After drying at 100° for 30 min the precipitates were transferred to weighed scintillation vials, ground to a fine powder with a glass rod, and the vial was reweighed. The solid was suspended in 10 ml of scintillation fluid containing 3.4% (w/v) Cab-o-sil [a finely divided silica as used by Cluley (1962)], 0.01% (w/v) POPOP and 0.4% (w/v) PPO in xylene and counted in a Nuclear Chicago Scintillation System 725. The channel-ratio method of Hendler (1964) was used to correct for extraneous quenching. No self-absorption occurred with quantities of BaCO_3 up to 50 mg, and all weights were kept below this. Recovery of $\text{NaH}^{14}\text{CO}_3$ put through the above procedure was 90% (range 86–94%).

Radioactive compounds

Sodium [$1\text{-}^{14}\text{C}$]acetate, sodium [$1\text{-}^{14}\text{C}$]propionate, sodium [$1\text{-}^{14}\text{C}$]butyrate, sodium [$2\text{-}^{14}\text{C}$]butyrate and $\text{NaH}^{14}\text{CO}_3$ were obtained from the Radiochemical Centre, Amersham, England.

Samples of infusion solutions were checked for radioactivity after isolation of the acids by silica-gel chromatography. Infusion solution (1 ml) plus 40 μ moles of the acid were dried at pH 10 and the acid was isolated and counted as described for rumen acids. The radioactivity of infusion solutions which contained $\text{NaH}^{14}\text{CO}_3$ was checked after isolation of the carbonate as BaCO_3 . Infusion solution (1 ml) plus 2 ml of 1% (w/v) NaHCO_3 were assayed after precipitation of the carbonate as BaCO_3 , as already described for CO_2 .

RESULTS

Pattern of rumen fermentation over a 24 h period

The results in Fig. 1 show the mean concentrations of VFA in the rumen of three sheep over the last 24 h of a 5-day feeding period. The mean acetic, propionic and butyric acid concentrations throughout the 24 h period were 61.5, 14.9 and 6.6 m-moles/l. respectively. The concentrations increased on feeding and maximum values were reached about 2 pm and maintained until 1–2 h after the last feed (approx. 9 pm). The molar proportions of acetic, propionic and butyric acids remained fairly constant throughout the 24 h period (Fig. 1). Infusions were made for 4 h from 1 pm since this allowed estimates of VFA production to be made at constant rumen concentrations.

Rumen pH

In all fifteen experiments made, the pH of the rumen contents remained fairly constant and ranged between 6.0 and 6.5; pH changes during the infusion period were small or absent.

Digestibility of ration

The digestibility and proximate analysis of the constituents of the ration were determined on the hourly feeding regimes for all sheep. Crude fibre and crude protein constituted 29.5% and 16.4% of the total dry matter in the lucerne respectively,

and the apparent digestibility of organic matter was 61%. The sheep were consuming 3230 kcal energy of which 56% (i.e. 1879 kcal) was apparently digestible.

Mean VFA concentrations during infusion experiments

Table 1 shows that there were differences in total VFA concentrations between experiments. However, these differences were small and no explanation can be offered. There was no significant difference in the percentage of the individual VFA in the rumen fluid in all experiments made.

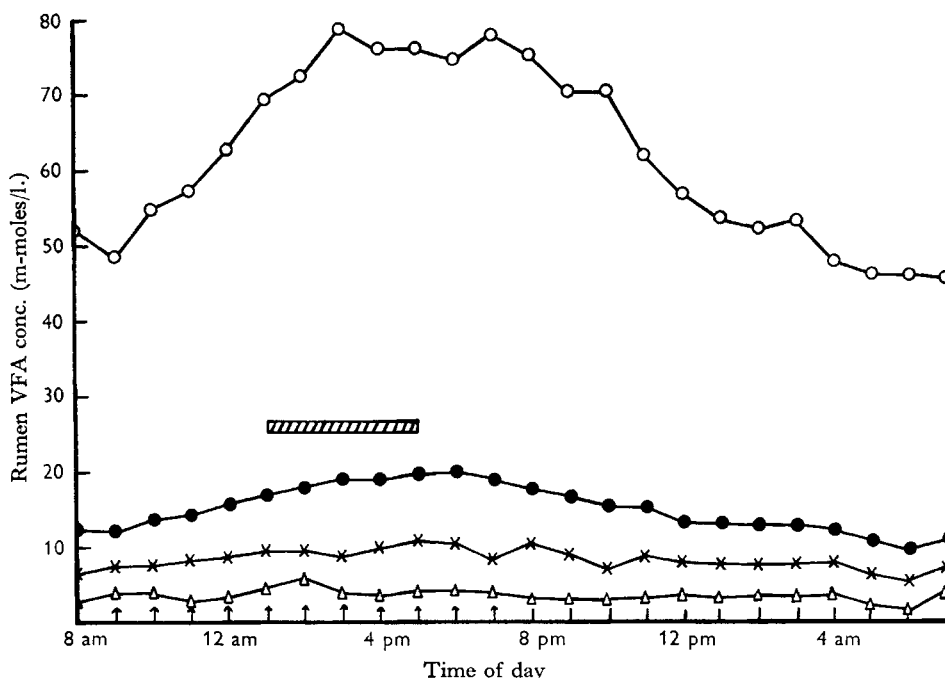


Fig. 1. Mean concentration of volatile fatty acids in the rumen of three sheep fed at hourly intervals from 8 am to 7 pm. ○—○, acetic acid; ●—●, propionic acid; ×—×, butyric acid; Δ—Δ, branched-chain and higher acids; ↑, indicates feeding times; ▨, infusion period.

Table 1. Concentrations of volatile fatty acids in pooled samples of rumen fluid from individual infusion experiments

(Individual results were obtained by pooling 1 ml portions from all samples obtained from one sheep during an infusion)

Infusate	No. of experiments	Total VFA (m-moles/l.)	Molar percentage of total VFA as*					
			Acetic acid	Propionic acid	Butyric acid	Iso-butyric acid	Iso-valeric acid	Valeric acid
[1- ¹⁴ C]Acetate	4	134 ± 6	68.6 ± 4.5	20.7 ± 2.3	7.5 ± 1.0	0.8 ± 0.3	0.9 ± 0.2	1.5 ± 0.3
[1- ¹⁴ C]Propionate	3	113 ± 1	68.2 ± 0.7	20.0 ± 0.6	8.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	2.0 ± 0.1
[1- ¹⁴ C]Butyrate	6	121 ± 9	68.4 ± 0.6	21.5 ± 1.0	7.1 ± 0.7	0.8 ± 0.3	1.0 ± 0.1	1.2 ± 0.1
NaH ¹⁴ CO ₃	2	144	69.2	19.0	8.0	1.0	1.0	1.8

* Mean values with their standard errors.

Radioactivity of rumen branched-chain and higher organic acids

The amount of radioactivity incorporated into the branched-chain and higher acids was low during the infusion of labelled acids, indicating that only small quantities of the latter acids were converted into the higher or branched-chain acids (Table 2).

Production rates of organic acids in the rumen

The specific activities of acetic, propionic and butyric acids during infusions of [$1-^{14}\text{C}$]acetate, [$1-^{14}\text{C}$]propionate or [$1-^{14}\text{C}$]- or [$2-^{14}\text{C}$]-butyrate are shown in Figs. 2, 3 and 4 respectively. The specific activities were constant from about 150 or 180 to

Table 2. *Specific activity ($\mu\text{C}/\text{mole}$) of organic acids in rumen fluid at the end of an infusion*

(Terminal samples were analysed from typical infusions of each labelled acid)

Infusate	Acetic acid	Propionic acid	Butyric acid	Branched-chain and higher acids
[$1-^{14}\text{C}$]Acetate	124	11	40	7
[$1-^{14}\text{C}$]Propionate	15	312	8	10
[$1-^{14}\text{C}$]Butyrate	37	27	432	13

Table 3. *Production rates of organic acids in the rumen of sheep*

Infusate	No. of experiments	Mean wt of sheep (kg)	Production rate* (m-moles/min)	Standard deviation for estimations in sheep†
[$1-^{14}\text{C}$]Acetate	4	37.0 (35-38)	4.23 ± 0.72	0.83
[$1-^{14}\text{C}$]Propionate	3	37.8 (37-39)	1.17 ± 0.18	0.12
[$1-^{14}\text{C}$]Butyrate	6	36.1 (35-39)	0.97 ± 0.12	0.19

* Mean values with their standard errors.

† Variation of calculated production rates using one of any estimation of specific activity in the same sheep (taken between 150 and 240 min of an infusion).

Table 4. *Radioactivity of rumen organic acids of sheep estimated under steady-state conditions and interconversion of one acid with another*

Infused acid	No. of experiments	Specific activity* ($\mu\text{C}/\text{mole}$)			% propionic acid produced from acetic acid	% butyric acid produced from acetic acid
		Acetic acid	Propionic acid	Butyric acid		
[$1-^{14}\text{C}$]Acetic	4	118 ± 20	8 ± 1	40 ± 14	4.5	16.9
					% acetic acid produced from propionic acid	% butyric acid produced from propionic acid
[$1-^{14}\text{C}$]Propionic	3	9 ± 2	429 ± 68	5 ± 1	3.2	0.9
					% propionic acid produced from butyric acid	% acetic acid produced from butyric acid
[$1-^{14}\text{C}$]Butyric	6	38 ± 11	13 ± 3	516 ± 63	3.4	14.7

* Mean values with their standard errors.

240 min after the start of an infusion. The mean specific activity ($\mu\text{C}/\text{mole}$) of the acid under investigation after the specific activity had reached a plateau compared with the rate of infusion of activity ($\mu\text{C}/\text{min}$) allowed estimates to be made of the production rates (m-moles/min) of the acids in the rumen (Table 3).

Effective production rates of VFA

Isotope dilution techniques as used in these investigations measure the amount of the acid that is entering the rumen pool of that compound and do not give any indication of the quantities being lost by conversion into other compounds. The studies

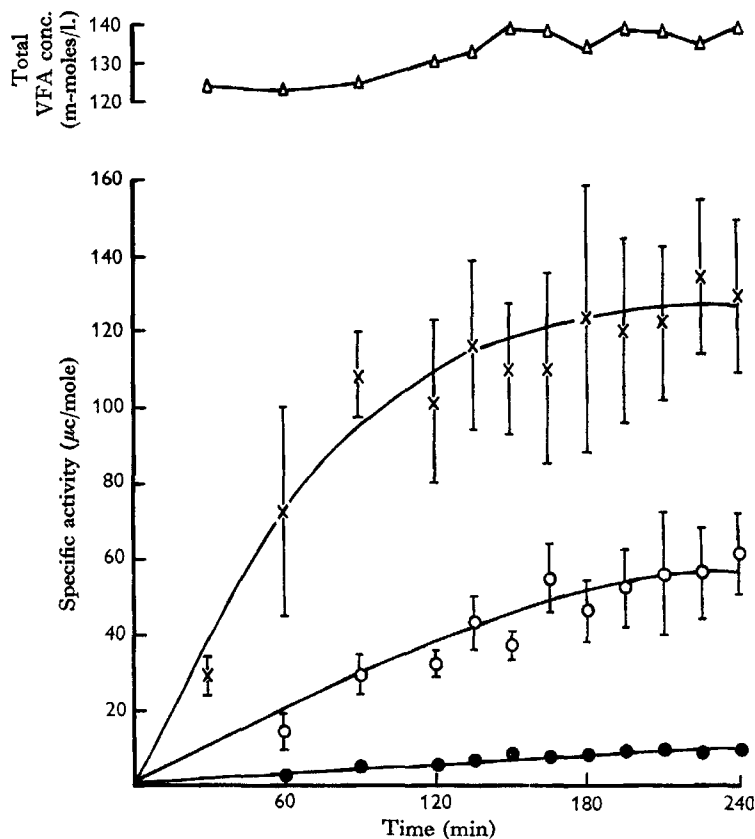


Fig. 2. Specific activities (sp.ac.) of rumen acetic, propionic and butyric acids during intraruminal infusions ($0.5 \mu\text{C}$, $1 \mu\text{mole}/\text{ml}$ per min) of [$1-^{14}\text{C}$]acetate. The concentration of total VFA is also shown. $\times-\times$, sp.ac. of acetic acid; $\bullet-\bullet$, sp.ac. of propionic acid; $\circ-\circ$, sp.ac. of butyric acid; $\Delta-\Delta$, total VFA concentration. Individual points are the means for four experiments. For acetic and butyric acid, the standard errors of the mean of four results have been included.

presented here indicate that considerable interconversions of VFA occur (Figs. 2, 3, 4), and therefore production rates are an overestimate of the quantities of the acids becoming available to the animal. A correction can be made for the rates of conversion of the acid under investigation into other acids in the rumen by comparing the specific activities of the individual acids after mixing is complete (Table 4), to obtain the

percentage of one acid converted into the other, and then multiplying the respective production rate by this percentage in order to obtain interconversion rate (Table 5). An example of this correction for the interconversion of acetic and butyric acids is as follows:

(1) Under steady-state conditions the mean specific activities of acetic and butyric acids during $[1-^{14}\text{C}]$ acetate infusions were 118 and $40 \mu\text{C}/\text{mole}$ (or 4.91 and $0.83 \mu\text{C}/\text{g}$ carbon) respectively (Table 4).

(2) The percentage of the butyric acid produced in the rumen arising from acetic acid is therefore $(0.83/4.91) \times 100 = 16.9$ (Table 4).

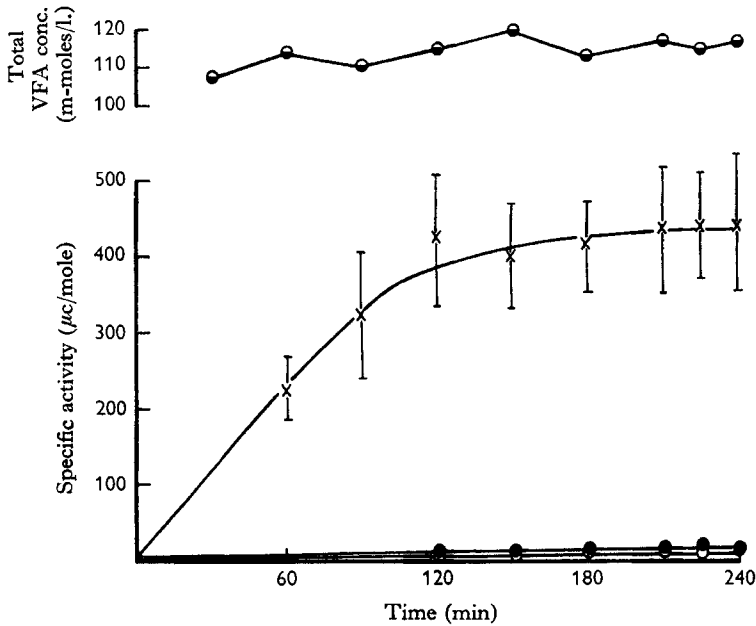


Fig. 3. Specific activities (sp.ac.) of rumen acetic, propionic and butyric acids during intraruminal infusions ($0.5 \mu\text{C}$, $1 \mu\text{mole}/\text{ml}$ per min) of $[1-^{14}\text{C}]$ propionate. The concentration of total VFA is also shown. \circ — \circ , sp.ac. of acetic acid; \times — \times , sp.ac. of propionic acid; \bullet — \bullet , sp.ac. of butyric acid; \ominus — \ominus , total VFA concentrations. Individual points are the means for three experiments. For propionic acid the standard errors of the mean of three results have been included.

(3) The rate of conversion of acetic into butyric acid is equal to the production rate of butyric acid multiplied by the percentage of the butyric acid produced from acetic acid, multiplied by 2 (assuming that 2 moles of acetic acid give rise to 1 mole of butyric acid) (Table 5), i.e. $0.97 \times 16.9 \times 2 = 0.33$ m-mole/min.

(4) In a similar way the rate of conversion of acetic acid into propionic acid may be estimated.

(5) The effective production rate is calculated as the measured production rate minus the sum of the rates of conversion of that acid into the other two major acids (Table 5).

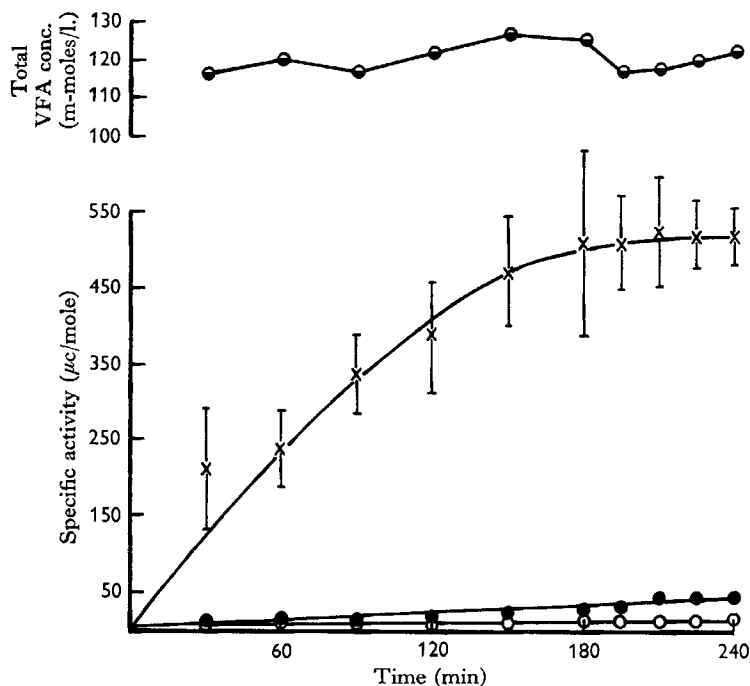


Fig. 4. Specific activities (sp.ac.) of rumen acetic, propionic and butyric acids during intraruminal infusions ($0.5 \mu\text{c}$, $1 \mu\text{mole/ml}$ per min) of $[1-^{14}\text{C}]$ - or $[2-^{14}\text{C}]$ butyrate. The concentration of total VFA is also shown. ●—●, sp.ac. of acetic acid; ○—○, sp.ac. of propionic acid; ×—×, sp.ac. of butyric acid; ●—●, total VFA concentration. Individual points are the means for six experiments. For butyric acid the standard errors of the mean of six results have been included.

Table 5. Calculation of effective production rates of volatile fatty acids in the rumen of sheep

Acid	Production rate (m-moles/min)	Rate of interconversion of VFA* (m-moles/min)					Effective production rate (m-moles/min)
		Acetic to propionic	Acetic to butyric	Propionic to acetic	Propionic to butyric	Butyric to acetic	
Acetic	4.23	0.05	0.33	—	—	—	3.85
Propionic	1.17	—	—	0.14	0.02	—	1.01
Butyric	0.97	—	—	—	—	0.31	0.64

* Interconversions were calculated assuming that:

- 1 mole acetic would give rise to 1 mole propionic acid or $\frac{1}{2}$ mole butyric acid,
- 1 mole butyric acid would give rise to 2 moles propionic acid or 2 moles acetic acid,
- 1 mole propionic acid would give rise to 1 mole acetic acid or $\frac{1}{2}$ mole butyric acid.

Daily production rates of VFA

The production rates measured in these investigations are maximum values. If it is assumed that production rates through 24 h vary proportionately with concentration, then estimates may be obtained for daily production rates by extrapolating from the mean concentrations of acetic, propionic and butyric acids over a 24 h period (see Fig. 1). The calculations are shown in Table 6. A comparison of the calculated

energy available from the three acids (1489 kcal/day) and the apparently digestible energy of the ration (1879 kcal/day) indicated that 79% of this energy was accounted for by VFA produced in the rumen.

Table 6. *Daily production rates of organic acids in the rumen of sheep*

Acid	Mean conc.* (m-moles/l.)	Mean calculated production† rate (m-moles/min)	Daily production (g)	kcal/day
Acetic	61.5	2.60	224.4	785.4
Propionic	14.9	0.63	67.3	333.8
Butyric	6.6	0.49	62.1	369.5
Total				1488.7

* Taken as the mean concentration of twenty-four samples taken over 1 day (see Fig. 1).

† Assuming that the production of VFA in the rumen varies proportionately with the concentration.

Oxidation of VFA

The radioactivity arising in rumen fluid and blood CO₂ was measured over the terminal stages of an infusion of ¹⁴C-labelled acid (five samples between 180 and 240 min) (Table 7). Considerable ¹⁴CO₂ always appeared in the two media, but in the experiments in which [1-¹⁴C]acetate or [2-¹⁴C]butyrate was used the specific activity of blood CO₂ was greater than that of the rumen CO₂. With [1-¹⁴C]propionate infusions, however, in one experiment the CO₂ in the rumen fluid was labelled to a greater extent than blood CO₂.

Table 7. *Contribution of rumen volatile fatty acids to total carbon dioxide output of sheep*

Infusate	Expt no.	Specific activity* (μc/g C)			Contribution of the oxidation of rumen acids to total CO ₂ output† (%)
		Infused acid	Blood CO ₂	Rumen CO ₂	
[1- ¹⁴ C]Acetate	G 10	7.4	1.3	0.6	17.6
[1- ¹⁴ C]Propionate	G 8	14.9	1.9	2.6	12.8
[1- ¹⁴ C]Propionate	G 7	12.4	1.6	1.2	12.9
[2- ¹⁴ C]Butyrate	G 1	9.0	1.2	0.5	13.3
[2- ¹⁴ C]Butyrate	G 2	8.5	1.3	0.7	15.3

* Specific activities of CO₂ were measured during the 3rd-4th h of infusion (five samples).

† Estimated by comparing the specific activities of blood CO₂ and the rumen acid labelled by infusion of tracer.

A comparison of the specific activities of acids in the rumen with that of the blood CO₂ gives a rough estimate of the contribution of the oxidation of acids produced in the rumen to total CO₂ output (Table 7). These figures are minimum figures since equilibration of the total body CO₂ had not occurred and the specific activity of circulating blood CO₂ was still increasing at the end of a 4 h infusion.

Because of the high incorporation of butyrate carbon into ketone bodies during intraportal infusions of [¹⁴C]butyrate (Annison, Leng, Lindsay & White, 1963) and

because of the possibility of non-enzymic decarboxylation of acetoacetate in blood *in vivo* or after sampling (Leng & Annison, 1964), [2-¹⁴C]butyrate was infused intraruminally in experiments in which the contribution of butyrate oxidation to total CO₂ output was examined. Under these circumstances any acetoacetate produced in the animal would be labelled mainly in the α -carbon atom (Annison *et al.* 1963) and decarboxylation of acetoacetate would not affect the result.

CO₂ fixation in the rumen

Since the rumen CO₂ became labelled during infusions of ¹⁴C-labelled VFA, the specific activity of the individual acids may be greater than expected if CO₂ fixation occurs to any extent. Fixation would tend to cause underestimation of production rates since specific activities of the acids would be increased. However, infusion of NaH¹⁴CO₃ into the rumen showed that very small amounts of ¹⁴C appeared in rumen acids (Table 8). There was a greater incorporation of ¹⁴C from NaH¹⁴CO₃ into propionic than into the other acids, indicating a small amount of CO₂ fixation. The results suggest that CO₂ fixation as a cause of error in these studies may be ignored.

Table 8. *Labelling of rumen acids during intraruminal infusion of NaH¹⁴CO₃*

(Infusion rate was 0.5 μ C, 1 μ mole/ml per min. The results are the means for two experiments)

Time (min)	Specific activity of rumen CO ₂ (μ C/mole)	Specific activity of blood CO ₂ (μ C/mole)	Specific activity of rumen VFA (μ C/mole)		
			Acetic acid	Propionic acid	Butyric acid
60	157	112	—	—	—
90	412	120	—	—	—
120	517	198	1.2	15.0	1.4
150	558	156	1.5	21.2	1.6
180	566	146	1.8	27.0	2.0
210	563	213	1.5	29.9	2.4
240	570	211	2.2	33.1	2.8
270	728	208	2.2	37.5	2.9
300	640	210	2.7	40.7	3.0

DISCUSSION

The purpose of the study now presented was more to explore the radioisotope dilution technique as a method for estimating the rates of organic acid production in the rumen than to provide a number of values for these rates. The results suggest that the method will be of value for making these measurements in sheep under various nutritional regimes, and that it will also be of considerable use in studies of the oxidation rates and contribution to energy metabolism of VFA, particularly of propionate and butyrate.

It can be seen from Figs. 1, 2, 3 and 4, that VFA levels remained fairly constant over the time-course of the infusion, particularly over the last 2 h when the measurements were made, which is necessary in any method attempting to measure production rates using isotope dilution. It was also found that the percentages of all organic

acids in the rumen of sheep were similar both between sheep and within the same animal, indicating that the hourly feeding regime used in these studies produced a fairly repeatable fermentation rate.

Both Annison (1965) and Warner (1964) have discussed the inherent difficulties of obtaining representative samples of rumen contents and suggest that the rates of production of acids are not uniform throughout the rumen. Results obtained with a single injection of labelled VFA introduced into the rumen tended to support this hypothesis, since samples of rumen contents obtained from various sites of the rumen after time had elapsed to allow complete mixing varied in specific activity by up to 20% (Leng & Leonard, unpublished observations), which agrees with the 20% variation within an animal of the specific activity of the acid under investigation (at constant specific activities) (see Table 3). The measurements of specific activities of VFA made between 150 and 240 min of an infusion in any animal showed a fairly high variation which was offset to some extent by the large number of samples examined at quite close intervals. The mean specific activity (after mixing) must represent the mean production rate of the acid in the rumen. If production rates vary throughout the rumen, regional variations may occur in the numbers or types, or in numbers and types of organisms responsible for fermentation, and also it suggests that there may be some layering of the food materials. Any attempt to mix rumen fluid uniformly by circulation pumps (Sutherland, Ellis, Reid & Murray, 1962; von Engelhardt, Hoeller & Hoernicke, 1963) may disturb the normal milieu within the rumen and may affect the rates and patterns of fermentation.

VFA, and in particular acetate, are readily oxidized by sheep tissues, and thus intraruminal infusions must label the animal's CO_2 pool. This raises the problem of recycling of ^{14}C through CO_2 fixation into VFA in the rumen. With any acid labelled by infusion of radioisotope there was considerable production of $^{14}\text{CO}_2$. However, it was indicated by intraruminal infusion of $\text{NaH}^{14}\text{CO}_3$ that the incorporation of $^{14}\text{CO}_2$ into rumen VFA would be negligible with the order of radioactivity in circulating CO_2 that occurred during infusions of ^{14}C -labelled VFA.

The site of production of $^{14}\text{CO}_2$ is difficult to assess, but if the oxidation only occurred in the body tissues then the blood CO_2 would be highly labelled and the rumen CO_2 would tend to become labelled by transport of HCO_3^- into the rumen in the saliva or across the rumen wall. With propionate, however, the specific activities of rumen and blood CO_2 indicated that to some extent oxidation (or decarboxylation) occurs in the rumen (Table 7).

Comparison of the specific activities of acetic, propionic and butyric acids with that of blood CO_2 during the terminal stages of infusions of individual acids allowed estimates to be made of the contribution of the oxidation of these acids to total respired CO_2 and therefore to the oxidative metabolism of the animal. The values in Table 7 indicate that they contribute considerably to the energy metabolism of the sheep. However, because specific activities of rumen and blood CO_2 were not the same, and since the specific activity of blood CO_2 had not reached equilibrium between the 3rd and 4th h of the infusion, an examination of the various experiments suggests that the contribution to total CO_2 output is underestimated by at least 20%. If it

is so, then more than 50% of the CO₂ respired by the fed animal arises from the oxidation of the major VFA produced in the rumen. No attempts have been made to correct the various figures for the ¹⁴CO₂ arising from the oxidation of acids other than those infused that have become labelled through interconversion. A more intensive investigation of VFA oxidation using intraruminal infusion of labelled acid is at present in progress (Leng, West & Annison, unpublished).

Difficulty is experienced in comparing the results obtained in these studies with results of others, since these are the first estimates using a continuous feeding regime. Gray *et al.* (1960) fed their animals on wheaten straw and recorded VFA concentrations of 57–67 m-moles/l., approximately half the levels obtained in these experiments. The estimated production rates were, however, approximately half those found in our studies. In the studies of Sheppard *et al.* (1959), only one animal was examined and the measurement of acetate production rate was made over a period of time in which the total VFA concentration was decreasing.

Extrapolation of production rates to daily production rates is subject to many errors since VFA concentrations (and presumably production rates) in the rumen are continuously changing in animals fed once or twice daily. With sheep, feeding usually occurs to only a small extent at night and therefore VFA levels vary considerably throughout a 24 h period. Assuming that production rates (effective) through 24 h vary proportionately with concentrations of the acids in the rumen, an overall daily production rate was calculated (Table 6). A comparison of the apparently digestible energy of the ration (1879 kcal) and the energy of the quantities of acids calculated to be produced in a day (1489 kcal) (see Table 6) indicates that the VFA produced in the rumen accounted for 79% of the apparently digestible energy of the ration. Although the production rates of VFA have been corrected for interconversion rates, these figures are probably overestimates of the quantities available for utilization by the body tissues since VFA may be converted into other compounds in the rumen. Little attention has been paid to the metabolism of VFA by rumen organisms, and it may be reasonable to suggest that a proportion of the acids produced in the rumen may be used for bacterial synthesis (i.e. fat, protein) and metabolism.

SUMMARY

1. Production rates of acetic, propionic and butyric acids in the rumen of sheep have been measured using isotope dilution techniques.
2. Animals were offered lucerne chaff (75 g) at hourly intervals between the hours of 8 am and 7 pm and given isotope infusions for 4 h from 1 pm.
3. The molar proportions of VFA in the rumen remained fairly constant throughout a 24 h period. VFA concentrations in the rumen increased on feeding and reached a plateau level around 2 pm and then remained fairly constant until 8 pm when a steady decline occurred. The VFA concentrations during infusion periods were fairly constant.
4. Infusions of sodium [¹⁻¹⁴C]acetate, sodium [¹⁻¹⁴C]propionate or sodium [¹⁻¹⁴C]- or [²⁻¹⁴C]-butyrate were each given in separate experiments at a rate per min of 0.5 μc,

1 μ mole/ml for 4 h. Samples of rumen fluid were taken at intervals over the infusion periods and the individual VFA were isolated and assayed for radioactivity. The specific activity of the infused acid was constant between 150 and 240 min after the infusion started. The production rates of acetic, propionic and butyric acids were 4.23 ± 0.72 , 1.17 ± 0.18 and 0.97 ± 0.12 m-moles/min respectively.

5. Comparisons of the specific activities of acetic, propionic and butyric acids during the terminal stages of an infusion of any one labelled acid allowed estimates to be made of the percentage interconversions of the acids. The rate of interconversion of the major VFA was estimated from a knowledge of the interconversion percentages and the production rates. From these figures the effective production rate of that acid was calculated. Effective production rates of acetic, propionic and butyric acids were 3.85, 1.01 and 0.64 m-moles/min respectively. From the average daily levels of the acids, and assuming that production rates vary proportionately with concentration, an average daily production rate was calculated, which indicated that approximately 79% of the apparently digestible energy of the ration could be accounted for by VFA production.

6. The incorporation of activity from the major VFA into the branched-chain and higher acids was low.

7. A comparison of the specific activities of blood carbon dioxide and rumen VFA in the terminal stages of an infusion indicated that 17.6, 12.9 and 14.3% of the animal's CO₂ output arose from the oxidation of acetic, propionic and butyric acids respectively.

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