Interconversions and Production of Volatile Fatty Acids in the Sheep Rumen

BY E. N. BERGMAN,* R. S. REID, MOIRA G. MURRAY, J. M. BROCKWAY AND F. G. WHITELAW
Rowett Research Institute, Bucksburn, Aberdeen

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1. Sheep fed at a constant rate were infused intraruminally with [1-^{14}C]-acetate, -propionate or -butyrate during 5hr. periods. 2. Volatile fatty acids were estimated in the rumen contents and steady-state conditions were obtained. 3. Of the butyric acid carbon 60% was in equilibrium with 20% of the acetic acid carbon, and 2–3g.atoms of carbon were interconverted/day. 4. Little interconversion took place between propionic acid, acetic acid or butyric acid. 5. The net production rates for acetic acid, propionic acid and butyric acid were 3·7, 1·0 and 0·7 moles/day respectively. 6. The production of volatile fatty acids accounted for 80% of the animal's energy expenditure.

Large quantities of volatile fatty acids, particularly acetic acid, propionic acid and butyric acid, are produced in the rumen by microbial fermentation of dietary carbohydrates and protein and are absorbed into the bloodstream mainly through the rumen wall (Barcroft, McAnally & Phillipson, 1944). The quantitative importance of the individual volatile fatty acids as contributors to the total metabolism of the whole animal has therefore been the subject of considerable interest and investigation (Pfander & Phillipson, 1953; Schambye, 1955; Armstrong, Blaxter & Graham, 1957; Annison & Lindsay, 1961, 1962; Bensadoun, Paladines & Reid, 1962; Bergman & Kon, 1964*b*; Lindsay & Ford, 1964).

Isotope-dilution methods have been used to measure turnover or entry rates of various metabolites in the whole animal. The method of Steele, Wall, de Bodo & Altszuler (1956), introduced for the study of glucose metabolism, has since been extended to the metabolism of free fatty acids (Armstrong *et al.* 1961), acetoacetic acid (Bergman, Kon & Katz, 1963; Bergman & Kon, 1964*a*,*b*) and the volatile fatty acids in ruminants (Annison & Lindsay, 1961, 1962; Lindsay & Ford, 1964; Sabine & Johnson, 1964). When measuring turnover rates, however, it can be difficult to relate the results to true production or utilization of the specific metabolite under investigation since some of the labelled metabolite may exist in equilibrium with other compounds (Steele *et al.* 1956). Thus Bergman *et al.* (1963) and Bergman & Kon (1964*a*,*b*) have shown that acetoacetate turnover rates actually can approximate total ketone-body turnover owing to an interconvertibility of acetoacetate with β-hydroxybutyrate. Further, Vande Wiele, MacDonald, Gurpide & Lieberman (1963) have calculated separate secretory rates for two androgenic hormones that are only partially interconvertible and their mathematical analysis has afforded expressions of the rates of interconversions as well. It seems clear therefore that turnover-rate measurements of an individual or specific metabolite may need corrections for interconversions between closely related molecules. This problem is especially pertinent to the study of individual volatile fatty acids in the ruminant animal.

* Present address: Department of Physiology, New York State Veterinary College, Cornell University, Ithaca, N.Y., U.S.A.

METHODS

Animals and diet

Two mature Scottish Blackface × Border Leicester cross-bred wethers were each fitted with a 2in. rumen cannula. They were confined in individual pens in a controlled environment room at 18±1° and thoroughly accustomed to handling. Both sheep were given 900g. of dried grass cubes each day from a moving belt so that the cubes were consumed at 2–5min. intervals over the entire 24hr. period (Murray, Reid & Sutherland, 1962). This method of continuous feeding has been shown to result in nearly constant concentrations of materials in the digestive tract (Portugal, 1963). Both animals gained about 5kg. in weight for the first 2 months of this continuous feeding but thereafter their body weights remained nearly constant at 55 and 52kg.

The composition of the diet (89% dry matter), expressed in terms of dry matter, was: protein (N × 6·25), 10·6%; ether extract, 7·3%; 'normal acid fibre', 34·3%; nitrogen-free extract, 39·7%; ash, 8·1%; gross energy, 4·23 kcal./g.; digestible energy, 3·08 kcal./g. Caloric values of the diet were determined by a bomb calorimeter.

Experimental procedures

On the day of an experiment the sheep was placed in a stall and continuously fed from the moving belt described above. A rumen pump (Sutherland, Ellis, Reid & Murray, 1962) was used to mix the rumen contents and to serve as a sampling device. In this manner the contents were relatively homogeneous and mixing was complete in 20–30 min.

After pumping the rumen contents for 30 min., 10 g. of polyethylene glycol and a priming dose of a ^{14}C-labelled volatile fatty acid dissolved in 100 ml. of mN-NaOH were injected in the rumen. Immediately a continuous infusion (28 ml./hr.) of the same ^{14}C-labelled fatty acid was begun and continued for 4–5 hr. The general procedure of isotope-dilution experiments has been described in detail by Bergman (1963). The acetate experiments were begun within 15 min. after the termination of [^{14}C]butyrate infusion, but propionate experiments were performed on separate days and at intervals of at least 1 week. The amounts of ^{14}C-labelled fatty acid used for the experiments were calculated by performing two preliminary trials, the average amounts injected being: 18 μc of propionate as a primer dose plus 8 μc/hr.; 43 μc of acetate plus 24 μc/hr.; 9 μc of butyrate plus 5 μc/hr. A negligible carrier content was present in all instances.

Samples of rumen contents for determination of polyethylene glycol and volatile fatty acids were collected every 20–30 min., immediately chilled to about 5°, centrifuged and acidified with H_2SO_4. Samples for determination of dry matter were collected at the beginning and end of each experiment and dried at 105° for 24 hr.

Chemical methods

Polyethylene glycol. The rumen volume was estimated after the injection of 10 g. of polyethylene glycol by the method of Hyden (1961).

^{14}C-labelled volatile fatty acid. High-activity [1-^{14}C]-butyrate, [1-^{14}C]acetate and [2-^{14}C]propionate were obtained commercially and purified before use by gas–liquid chromatography (James & Martin, 1952). Specific activities after a second chromatographic separation were all within ±3% of that found after the first purification.

Estimation of volatile fatty acids. The fatty acids were titrated under CO_2-free conditions after steam-distillation in a Markham still. An excess of NaOH was added and the entire 150 ml. of distillate was evaporated on a steam bath to 0·25 ml. Acetic acid, propionic acid and butyric acid were then separated, titrated and collected by the gas–liquid-chromatographic method of James & Martin (1952). Valeric acid and higher acids comprised less than 2% of the total and therefore were omitted from the calculations.

Phenolphthalein was used as the indicator in the gas–liquid chromatogram since a colourless solution was necessary for liquid-scintillation counting. This necessitated redesigning the titration cell so that the gas bubbles were not seen' by the photocell. The principle used was that of a micro gas pump where the gas stream forced the water to flow through a circular glass tubing located between the photocell and light source. Clear separations of individual acids were obtained and recoveries of fatty acids after both steam-distillation and chromatography were 97%. When [^{14}C]-acetate, -propionate or -butyrate was added, less than 1% of the dose was recovered in each of the other two fractions.

The individual fatty acids, collected from the gas–liquid chromatogram, were evaporated to a volume of 2·0 ml. and assayed for radioactivity with a refrigerated liquid-scintillation counter (Bergman *et al.* 1963). The solutions were transferred quantitatively to the counting vials by rinsing with 8 ml. of scintillation fluid. The efficiency of counting was about 40% and the specific activities were expressed as μc/g.atom of carbon.

Rumen carbon dioxide. About 6 ml. of rumen contents was placed in the outer chamber of a 50 ml. Warburg-type flask and acidified with 0·5 ml. of 10 N-H_2SO_4. The evolved CO_2 was trapped in CO_2-free 2 N-NaOH and precipitated as $BaCO_3$ (Bergman & Sellers, 1960). The $BaCO_3$ was washed with 2 M-sodium acetate, reconverted into CO_2 and again trapped in 2 N-NaOH for assay of radioactivity (Bergman & Kon, 1964*a*).

Calorimetry

Measurements of the heat output of the sheep were carried out over 20–24 hr. in the large gradient-layer calorimeter described by Pullar (1958). The sheep underwent preliminary training before the actual measurements of heat loss reported below were made. Conditions inside the calorimeter were kept as close as possible to those experienced by the sheep in the laboratory except that the sheep had to be confined to a metabolism cage. Water was available *ad lib.* and the ration of grass cubes was supplied from the continuous-belt feeder. Total recorded heat losses were corrected for the heat production (48 kcal./day) of the electric motor that powered the feeder.

RESULTS

Experimental conditions. Twelve experiments were performed on the continuously fed sheep, whose rumen contents were artificially and continuously mixed. Table 1 summarizes the experimental data for labelled fatty acids used, rumen volumes and concentrations and molecular proportions of rumen volatile fatty acids. Detailed results typical of the individual acetic acid, propionic acid and butyric acid experiments are shown in Fig. 1. Nearly constant specific activities, indicative of a steady-state condition, were obtained after 2–3 hr. of infusion and all calculations were based on this period of the experiment.

The concentration of fatty acids, as shown in Fig. 1, tended to increase slightly with time (mean 1·5%/hr.; range 0·6–2·2%/hr.), but the rumen volume and percentage dry matter appeared to show concomitant increases and decreases respectively (0·5–1·0%/hr.). These minor changes prob-

Table 1. *Mean experimental values obtained when sheep were continuously fed and the rumen contents mechanically mixed*

Expts. 5P, 10A and 10B were performed on sheep 2 and the others on sheep 1. The molecular proportions and concentrations of the rumen volatile fatty acids are mean values obtained over the 4–5 hr. period of the experiment. Rumen volumes and dry matter were measured at the beginning of each experiment.

Expt. no.	^{14}C-labelled fatty acid infused	Rumen volatile fatty acids: Mol. prop. (%) Acetic acid	Propionic acid	Butyric acid	Total (m-moles/l.)	Rumen volume (l.)	Dry matter (%)
3P	[2-^{14}C]Propionate	70	17	13	99	5·2	
5P	[2-^{14}C]Propionate	64	21	15	111	4·9	10·2
7P	[2-^{14}C]Propionate	67	21	12	108	5·7	8·7
9P	[2-^{14}C]Propionate	68	18	14	117	6·0	9·0
Mean		67	19	14	109	5·5	9·3
4A	[1-^{14}C]Acetate	70	17	13	93	5·6	7·9
6A	[1-^{14}C]Acetate	69	20	11	104	6·1	8·1
8A	[1-^{14}C]Acetate	64	20	16	116	5·7	8·5
10A	[1-^{14}C]Acetate	68	18	14	114	4·9	9·6
Mean		68	19	13	107	5·4	8·5
4B	[1-^{14}C]Butyrate	70	18	12	87	5·6	8·1
6B	[1-^{14}C]Butyrate	68	20	12	95	5·7	8·4
8B	[1-^{14}C]Butyrate	63	21	16	113	5·7	9·1
10B	[1-^{14}C]Butyrate	69	18	13	107	4·7	9·9
Mean		68	19	13	101	5·6	8·9

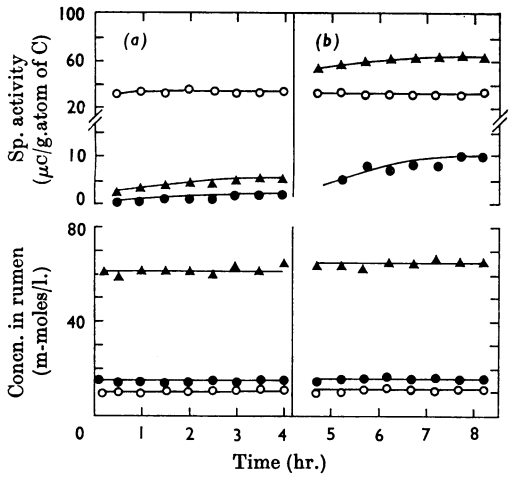


Fig. 1. Concentrations and specific activities of butyric acid (○), acetic acid (▲) and propionic acid (●) in the rumen of sheep infused with (*a*) [1-^{14}C]butyrate (Expt. 4B) and (*b*) [1-^{14}C]acetate (Expt. 4A). The butyrate primer dose was 8·5 μc and the infusion rate 5·6 μc/hr. The acetate primer dose was 41 μc and the infusion rate 27 μc/hr.

ably reflect only a slightly decreased removal of fatty acids due to decreased rumen absorption or decreased outflow to the lower digestive tract.

The molecular proportions of the fatty acids were fairly constant during each experiment and remained within ±5% of the mean value reported (Table 1).

Interconversions of the volatile fatty acids. Table 2 lists the mean percentages of the ^{14}C found in each of the three fatty acid fractions, together with their specific activities, during the last 1–2 hr. of the infusion of labelled fatty acids. The ^{14}C-transfer ratios (Table 2) are a measure of the percentages of the individual fatty acids derived from the precursor. Considerable interconversions occurred between acetic acid and butyric acid; 61% (range 51–66%) of the butyric acid carbon was derived from acetic acid, and 20% (range 15–28%) of the acetic acid carbon originated from butyric acid. Interconversions between propionic acid and the acetic acid and butyric acid fractions were much less, but were greater than could be accounted for by ^{14}C contamination (less than 1%) during chromatography.

The amount of radioactivity found in rumen carbon dioxide accounted for less than 2% of the rumen carbon dioxide production.

Rates of production and interconversion of volatile fatty acids. The 12 experiments were divided into four groups and are listed in Table 3. Each group consisted of one acetic acid, one propionic acid and one butyric acid experiment, with each experiment having comparable rumen

Table 2. *Recovery of radioactivity in the rumen volatile fatty acids in the steady state during intraruminal infusions of* ^{14}C*-labelled volatile fatty acids*

Specific activities were adjusted for an infusion rate of 100 μc/hr. for ease of comparison between experiments. The ^{14}C-transfer ratio is defined as the ratio of the specific activity of the specified volatile fatty acid to that of the volatile fatty acid infused.

Expt. no.	^{14}C-labelled fatty acid infused	Radioactivity (% of ^{14}C recovered)			Sp. activity (μc/g.atom of C)			100 × ^{14}C-transfer ratio		
		Acetic acid	Propionic acid	Butyric acid	Acetic acid	Propionic acid	Butyric acid	Acetic acid	Propionic acid	Butyric acid
3P	[2-^{14}C]Propionate	9	87	4	26	719	29	3·6		4·0
5P	[2-^{14}C]Propionate	10	85	5	44	642	50	6·8		7·7
7P	[2-^{14}C]Propionate	9	88	3	28	587	31	4·8		5·4
9P	[2-^{14}C]Propionate	5	93	2	18	660	15	2·7		2·2
Mean		8	88	4	29	652	31	4·5		4·8
4A	[1-^{14}C]Acetate	81	4	15	236	39	119		16	51
6A	[1-^{14}C]Acetate	77	7	16	241	50	153		21	63
8A	[1-^{14}C]Acetate	73	3	24	262	24	172		9	66
10A	[1-^{14}C]Acetate	77	3	20	273	31	169		11	62
Mean		77	4	19	253	36	153		14	61
4B	[1-^{14}C]Butyrate	27	4	69	91	33	616	15	5·3	
6B	[1-^{14}C]Butyrate	39	3	58	128	26	562	23	4·7	
8B	[1-^{14}C]Butyrate	33	4	63	122	30	425	28	7·0	
10B	[1-^{14}C]Butyrate	30	2	68	112	16	677	17	2·4	
Mean		33	3	64	113	26	570	20	4·8	

Table 3. *Production rates of volatile fatty acids in the rumen*

All values were calculated from the results of Table 2. Experimental details are given in the text.

Expt. no.	Volatile fatty acids (moles/day)						
	Gross production or entry rate			Net production rate			
	Acetic acid	Propionic acid	Butyric acid	Acetic acid	Propionic acid	Butyric acid	Total
3P, 4A, 4B	5·1	1·11	0·97	4·2	0·99	0·64	5·8
5P, 8A, 8B	4·6	1·25	1·41	3·2	0·90	0·92	5·1
7P, 6A, 6B	5·0	1·36	1·07	3·9	1·19	0·58	5·6
9P, 10A, 10B	4·4	1·21	0·89	3·5	1·13	0·58	5·2
Mean	4·7	1·23	1·05	3·7	1·05	0·68	5·4

fatty acid concentrations and molecular proportions (see also Table 1).

The gross production or entry rates, given in Table 3, were calculated by dilution of specific activity and are not corrected for any ^{14}C interconversions. The net production rates, however, were calculated by the use of a three-compartment model, illustrated in Scheme 1. This three-compartment model was patterned, in general, after the two-compartment model of Vande Wiele *et al.* (1963) for the secretion and interconversion of two androgenic hormones. The solution of the model shown in Scheme 1 was attained by a series of ten simultaneous equations as follows:

$$S_P = P_P + R_{PA} - R_{AP}$$

$$S_A = P_A + R_{AP} + R_{AB} - R_{PA} - R_{BA}$$

$$S_B = P_B + R_{BA} - R_{AB}$$

$$F_P + R_{AP}.a_A^P = (R_{PA} + P_P).a_P^P$$

$$R_{AP}.a_A^A = (R_{PA} + P_P).a_P^A$$

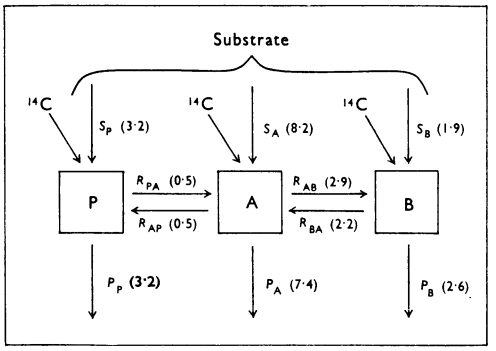
$$R_{PA}.a_P^P = (R_{AP} + P_A + R_{AB}).a_A^P$$

$$F_A + (R_{PA}.a_P^A) + (R_{BA}.a_B^A) = (R_{AP} + P_A + R_{AB}).a_A^A$$

$$R_{BA}.a_B^B = (R_{AB} + P_A + R_{AP}).a_A^B$$

$$R_{AB}.a_A^A = (R_{BA} + P_B).a_B^A$$

$$F_B + (R_{AB}.a_A^B) = (R_{BA} + P_B).a_B^B$$



Scheme 1. Three-compartment model used for the evaluation of production and interconversion of volatile fatty acids in the rumen by labelling with negligible quantities of highly ^{14}C-labelled fatty acids. P, Propionate; A, acetate; B, butyrate; S_P, S_A and S_B, 'direct secretory rates' of propionate, acetate and butyrate respectively; P_P, P_A and P_B, net production rates of propionate, acetate and butyrate respectively. R_{PA}, R_{AP}, R_{AB} and R_{BA}, rates of interconversion or transfer between the compartments indicated. The numbers in parentheses are calculated from the results obtained in the rumen of continuously fed sheep eating 900g. of dried grass cubes/day and are in terms of g.atoms of carbon/day.

where F_P, F_A and F_B are the infusion rates of propionate, acetate and butyrate respectively (μc/hr.), a_A^P is the specific activity (μc/g.atom of carbon) of acetate when propionate is infused and the remaining symbols are as defined in Scheme 1.

A complete solution of the three-compartment model for the interconversions and production of volatile fatty acids is also given in Scheme 1. The values are the means of four experiments on each acid. The transfer of carbon between propionic acid and acetic acid was small, but substantial interconversion was present between acetic acid and butyric acid.

The net production rates are the best estimate of production of volatile fatty acids in the rumen since in them allowance has been made for interconversions between the three fatty acids. The mean net production rate of propionic acid was only 14% lower than its gross rate, but the mean net rates for acetic acid and butyric acid were 21 and 35% lower than their respective gross rates (Table 3).

Estimated daily energy intakes and expenditures. These results are summarized in Table 4. Net production of volatile fatty acids, in terms of kcal./day, accounted for 45 and 62% of the gross energy intake and digestible energy intake respectively. When compared with the sheep's daily heat output or energy expenditure, with the animal confined to a metabolism cage in a calorimeter, the corresponding value was 82%.

Table 4. *Estimated daily energy intakes and expenditures in sheep*

Numbers in parentheses indicate the numbers of determinations of results given as means ± S.E.M.

	Energy (kcal./day)	Total volatile fatty acids (% of energy)
Gross energy intake	3341	45
Digestible energy intake	2446	62
Energy expenditure*	1844±41 (5)	82
Net volatile fatty acids produced:		
Acetic acid	773±41 (4)	
Propionic acid	386±19 (4)	
Butyric acid	357±35 (4)	
Total	1516±30 (4)	

* Direct calorimetry.

DISCUSSION

The results of the present studies confirm previous findings that two major pathways are involved in the formation of rumen volatile fatty acids (Jayasuriya & Hungate, 1959; Van Campen & Matrone, 1960; Baldwin, Wood & Emery, 1962). Acetic acid and butyric acid were to a considerable extent interconvertible, with a mean of 61% of the butyric acid carbon in equilibrium with about 20% of the acetic acid carbon. The smaller incorporation of acetic acid and butyric acid ^{14}C into propionic acid or vice versa probably represented randomization through the tricarboxylic acid cycle (Weinman, Strisower & Chaikoff, 1957), although some propionic acid carbon could be transferred to acetic acid via the glyoxalate pathway or acrylate pathway (Baldwin *et al.* 1962). A small amount of ^{14}C therefore would be expected to be lost as $^{14}CO_2$ during the transfer from [1-^{14}C]acetate or [1-^{14}C]butyrate to propionate, but relatively little $^{14}CO_2$ would appear during the transfer from [2-^{14}C]propionate to acetate or butyrate.

Sheppard, Forbes & Johnson (1959) and Gray, Jones & Pilgrim (1960) measured the production of volatile fatty acids in the rumen by measuring the decline in specific activity after an intraruminal injection of a ^{14}C-labelled fatty acid. Gray *et al.* (1960) observed that interconversions between acids had occurred and their calculated production rates could only be considered as gross entry rates. In the present study, the mean net production rates of volatile fatty acids in the rumen were 3·7, 1·0 and 0·7moles/day for acetic acid, propionic acid and butyric acid respectively when the sheep were eating 900g. of dried grass cubes/day. These rates were calculated by making allowances for fatty acid interconversions and totalled 5·4moles/day as

compared with the gross rate of 7·0moles/day. This is still probably an overestimate of true fatty acid production, however, since incorporation into amino acids and microbial protein undoubtedly occurred. Concentrations of free amino acids in rumen fluid are small (less than 0·03μmole/l.) but their rate of turnover appears to be rapid (Portugal, 1963). It seems unlikely that these are major quantitative factors, however, since protein constitutes only a small portion of the nutrient intake. The appearance of ^{14}C in rumen carbon dioxide was small and accounted for less than 2% of the rumen carbon dioxide production.

Previous studies on the production of volatile fatty acids in the rumen have been reviewed by Blaxter (1962) and by Warner (1964). Values obtained in sheep varied from 1 to 15 moles/day, with most values in the range 1–4 moles/day. Sutherland (1963), using the Carroll & Hungate (1954) technique of incubating rumen contents *in vitro*, together with measurements of rumen volume, reported that sheep produced 3·7moles of volatile fatty acids/day when fed on a diet similar in both quantity and quality to that of the present study. The higher protein content of the diet (16·4% compared with 10·6% in the present work), however, could explain some of the difference in results with the present experiments. It is difficult to compare the present results on the production of volatile fatty acids with the whole-animal acetate entry-rate measurements of Annison & Lindsay (1961, 1962) and Lindsay & Ford (1964) since, as shown by these investigators, endogenous acetate release undoubtedly accounted for a portion of the total entry rate. In addition, rumen epithelium and liver probably metabolize substantial quantities of the fatty acids before they appear in the peripheral blood.

The results in Table 4 indicate that the production of volatile fatty acids accounted for as much as 45 and 62% of the gross and digestible energy intakes respectively, and 82% of the total heat loss or energy expenditure of the animal. The total energy expenditure (1844kcal./day) of the animal was less than the digestible energy intake (2446kcal./day), since the latter includes such energy losses as methane in the eructated gases and nitrogenous compounds in the urine. In addition, the animal had to be confined to a metabolism cage during calorimetric measurements. The experiments emphasize, nevertheless, the dependence of the ruminant animal on volatile fatty acids for its metabolic requirements.

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