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Parameters of Rumen Fermentation in a Continuously Fed Sheep: Evidence of a Microbial Rumination Pool

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The feed and feces of a continuously fed sheep were analyzed for carbon, hydrogen, and nitrogen, with oxygen as the remainder. The daily feed-feces weight difference was used as the reactant in an equation representing the rumen fermentation. The measured products were the daily production of volatile fatty acids (VFA), CH_4, CO_2, and ammonia. The carbon unaccounted for was assumed to be in the microbial cell material produced in the rumen and absorbed before reaching the feces. The ratio of C to H, O, and N in bacteria was used to represent the elemental composition of the microbes formed in the rumen fermentation, completing the following equation:

$$C_{20.03}H_{36.99}O_{17.406}N_{1.345} + 5.65\ H_2O \rightarrow \underset{\text{VFA}}{C_{12}H_{24}O_{10.1}} + 0.83\ CH_4 + 2.76\ CO_2 + 0.50\ NH_3 + \underset{\text{microbial cells absorbed}}{C_{4.44}H_{8.88}O_{2.35}N_{0.785}}$$

With C arbitrarily balanced and O balanced by appropriate addition of water, any error is reflected in the H. The H recovery was 98.5%. The turnover rate constant for rumen liquid equilibrating with polyethylene glycol (PEG) was 2.27 per day. Direct counts and volume measurements of the individual types of bacteria and protozoa in the rumen were used to calculate the total microbial cell volume in the rumen, not equilibrating with it. The dry matter in the rumen (582 g) and the nitrogen content (12.05) of the microbes in the rumen were estimated, the latter constituting 85% of the measured N in the rumen. Calculations for rumen dry matter and nitrogen turning over at the PEG rate introduce big discrepancies with other parameters; a rumination pool must be postulated. Its size and composition are estimated. Arguments are presented to support the view that dry matter and some of the microbes, chiefly the protozoa, do not leave the rumen at the PEG rate. One experiment with the same sheep fed twice daily showed significantly less production of microbial cells than did the continuous (each 2 hr) feeding. Analysis of the microbial cell yield suggests that, on the basis of 11 mg of cells per adenosine triphosphate molecule, a maximum of six adenosine triphosphate molecules could have been formed from each molecule of hexose fermented.

The steps in analyzing a microbial ecosystem can be formulated as (i) describing the kinds and numbers of organisms concerned, (ii) identifying what they do, and (iii) observing how fast they do it. A complete description is kinetic, involving the rates of component processes and of the whole.

Steps i and ii have been practiced extensively at numerous laboratories over the world, and many rumen microbial species have been identified (2, 4). Their activities in the rumen have been inferred from the characteristics of the pure cultures and in a few instances (14, 15) have been investigated experimentally. The rates of many rumen activities have been measured in pursuance of step iii.

One criterion for the completeness and precision of a kinetic analysis of an ecosystem is the magnitude of the discrepancy between measured component rates, algebraically summed, and the measured rates of the total. This determination is

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necessary also for computer simulations. For the rumen, this involves measurement of microbial activities and their integration with host functions, in particular with the amount and kind of material disappearing during passage of food, and the turnover rates of the materials in the rumen. Such an analysis of the rumen is attempted, based on the continuous fermentation model (13).

Since constancy of the rumen increases its resemblance to the model, the sheep was fed at frequent evenly spaced intervals. For comparison, some measurements were made on the same animal fed twice daily.

MATERIALS AND METHODS

Experimental animal. From February until December, 1969, a 55-kg Corriedale wether with a permanent rumen fistula was fed from an automatic feeder set to supply 90 g of air-dried alfalfa pellets at 2-hr intervals. In January, 1970 the same daily ration [976 g (dry wt) from the same batch of alfalfa] was fed in two portions of 540 g each at 7:30 and 19:30. Water was available at all times.

Rumen contents were obtained through a glass tube (20-mm inside diameter) to which suction was applied. Prior to sampling, the contents were thoroughly mixed. Rumen liquid and small particles were obtained by inserting the perforated tube previously described (11) and withdrawing samples from its interior by suction.

Analytical methods. Feces were collected between 14 and 24 March 1969. A fixed proportion of each day's collection was dried at 65 C, pooled, thoroughly mixed, and sampled for the various analyses. The average dry matter in the feed was 90.06% and in feces 41.0%. The crude protein content (nitrogen × 6.25) of the pooled sample of feed or feces was estimated by the Kjeldahl technique. The Kjeldahl values on rumen contents do not include ammonia-N because ammonia was lost during drying as the material became alkaline due to loss of CO_2. Ammonia-N was determined by the Conway method, by using the supernatant fluid of freshly centrifuged rumen samples. Dissolved non-ammonia-N was estimated by Kjeldahl analysis of supernatant fluid, obtained by centrifugation of fresh rumen fluid at 25,000 × *g* for 30 min and dried before analysis.

Cellulose was determined with 80% acetic acid and concentrated HNO_3 (21), hemicellulose with 12.5% HCl and 0.05-N $KBrO_3$ (21), water-soluble carbohydrate by the anthrone method (27), and ash by conventional techniques. The *p*H was estimated with indicator paper and checked with a Beckman Zeromatic SS-3 *p*H-meter. Elementary analyses of feed and feces for carbon, hydrogen, and ash were performed by Galbraith Laboratories, Inc.

Polyethylene glycol application and analysis. Polyethylene glycol (PEG) was used as a marker for several measurements of the rumen turnover rate constant in the continously fed animal and for one measurement in the twice daily system in January, 1970.

PEG (10 g in 200 ml of water) was added to the rumen at 9:15 for the 2-hr feeding, and samples of rumen fluid were collected after 2, 3, 4, 6, 7, and 8 hr. For the twice daily feeding, the same amount was given immediately before feeding. Rumen samples were centrifuged and the PEG in the supernatant fluid was analyzed by the technique of Hyden (16). Rumen volume and rate of passage (turnover) were obtained from the linear regression equation for the ln PEG concentration against time.

Measurement of fermentation activity. Volatile fatty acids (VFA) were determined by gas chromatography (Aerograph model 600-D, FFAP column) of rumen fluid supernatant (30,000 × *g*) passed through a 0.45-μm membrane filter (Millipore Corp.) and acidified with orthophosphoric acid.

Production rates of VFA and ammonia were determined by the zero-time method (3, 13) with carbon dioxide in the gas phase. Methane production was estimated by incubating a sample of rumen contents anaerobically for 1 hr in a stoppered container in the water bath (39 C). The gas produced was allowed to escape into a syringe, measured, and analyzed with a thermal conductivity gas chromatograph provided with a silica gel column. For rates of carbon dioxide production from organic C (not bicarbonate), the carbon dioxide above the sample was displaced with N_2 just before the start of the measurement. At the end of the experiment, the culture was killed with sulfuric acid, inserted through the rubber stopper, which also released all CO_2 from bicarbonate. The acid was added to a control before incubation. Control and experimental tubes were equilibrated at room temperature, and the excess gas was allowed to escape into the measuring syringe. The carbon dioxide remaining in solution when the initial and final gas volumes were measured was estimated from the solubility coefficient of carbon dioxide at room temperature and added to the measured excess gas.

Microbial counts. Samples of rumen contents were preserved by adding one volume of 8% formaldehyde. This 2× dilution was further diluted 40×, a little crystal violet was added, and the bacteria were counted in a Petroff-Hauser counting chamber under oil immersion at ×1,000 magnification.

Protozoa were identified according to Dogiel's monograph (4). Individuals of different species were counted separately as follows (25): 1 ml of a formaldehyde-diluted sample was further diluted to 12 ml with 10 ml of water or Lugol's iodine solution and 1 ml of glycerol. The solution was mixed by syringe, and 0.05 ml was placed on a microscope slide. The largest particles of hay were removed with a thin pin, and the sample was covered with a cover glass. All protozoa in the drop were counted.

The rumen microbes were assumed to have a specific gravity of 1.1 and to contain 10% dry matter. An average nitrogen content of 10.5% was assumed for the bacteria (12) and 8% for the protozoa.

RESULTS

The results of proximate and elemental analyses of feed and feces are collected in Table 1.

The nitrogen content (2.41%) of the alfalfa

TABLE 1. *Daily disappearance of feed components (from analyses of feed and feces)*

Component	Amt consumed (g)	Amt in feces (g)	Difference	
			g	g atoms
Protein (N × 6.25)	147.3	29.3	118	
Cellulose	262	126	136	
Hemicellulose	126	64	62	
Water-soluble carbohydrates	111	3	108	
Other carbohydrates (by difference)	154.0	28	126	
Lipid (assumed value)	25.4	11.4	14	
Lignin (assumed value)	78	78	0	
Ash	72.3	29.3	43	
Total	(976)	(369)	(607)	
Carbon	411	170.64	240.36	20.03
Hydrogen	59.3	22.31	36.99	36.99
Nitrogen	23.52	4.69	18.83	1.345
Ash	72.3 (54.3 corrected)	29.3 (21.96 corrected)	43	
Oxygen (by difference)	427.88	149.4	278.48	17.405
Total	(976)	(369)	(607)	

pellets was close to that reported for 15% protein (2.4% N) alfalfa (Dehydrated Alfalfa, Assay Report, 3rd ed. American Dehydrators Ass., Kansas City, Mo. 64112). The mineral percentages given in that report were used to predict the ash expected in our material. A value of 6.45% ash was calculated, as compared to the found value of 6.94% for our alfalfa sample. The usual analytical values for ash differ from the actual mineral content because some elements, e.g. sulfur, take up oxygen in the process of ashing. From the mineral analyses of alfalfa (American Dehydrators Ass.), the amount of oxygen taken up during ashing was estimated to be 25% of the final ash weight. The corrected ash value used was 4.84%. The oxygen value in Table 1 was the difference between 100% and the summed percentages of C, H, N, and corrected ash.

The results of all PEG experiments to determine the turnover rate constant and the volume of the rumen contents, calculated from the PEG results, are shown in Table 2 for the 2-hr feeding experiments. The results of these experiments on the 2-hr regime were pooled, and a linear regression was calculated from the ln of all values of PEG concentration against time, giving the first order relationship in Fig. 1, with the equation

$$\ln \text{PEG concentration} = -0.0946 \text{ g/[(hour} \times \text{liter)} \times \text{time]} + \ln 2.65 \text{ g/liter} \quad (1)$$

The turnover rate constant of −0.946 per hr indicates that the PEG-containing volume turned over 0.0946 per hr; the volume entering and leaving the rumen in 10.6 hr was equal to the volume in the rumen; the rumen PEG volume turned over 2.27 times per day.

TABLE 2. *Results of polyethylene glycol (PEG) experiments*[a]

Date	Dilution rate constant per hr	Rumen vol from initial PEG concn (liters)
21 February 1969	−0.0896	3.51
3 March 1969	−0.1083	3.06
24 March 1969	−0.1148	3.15
18 April 1969	−0.0977	3.26
26 May 1969	−0.1536	3.57
4 June 1969	−0.1166	3.55

[a] PEG was added at 9:15.

At the initial concentration of 2.65 g/liter, 10 g of PEG was contained in 3.77 liters. This has been used as the average volume of the rumen liquid into which PEG diffused. PEG does not enter the microbial cells or the ruminant cells lining the gut. We have assumed that ingested plant cells are killed in the rumen, permitting PEG to enter. In the insoluble plant materials, PEG probably does not reach the same concentration as in the liquid, but since the extent of PEG exclusion from nonviable material is unknown and its neglect introduces relatively little error (see below for its estimation), it is assumed in this study that PEG equilibrates with all nonliving material but not with living particulate matter. It is also assumed that, because of the

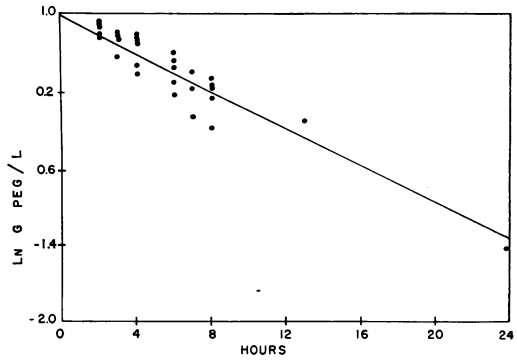

FIG. 1. *Regression of ln polyethylene glycol concentration against time, including results of all polyethylene glycol experiments.*

continuous nature of the 2-hr feeding system, all bacterial cells observed microscopically in the rumen were either alive or, if dead, had the same composition as live cells.

The counts of protozoa in the liquid and small particle (LSP) material removed through the sampling tube, and in the total rumen contents, are shown in Table 3 for the 2-hr feeding, and the bacterial counts are tabulated in Table 4. From the size and number of the microbial cells in the LSP material, their volume was estimated (Table 5) at 197.6 μliters/ml of rumen contents. Table 5 summarizes also the results of the microbial counts for the 12-hr feeding. The PEG-containing volume is 802.4 μliters/ml, and the total rumen volume is 4.7 liters.

The calculated microbial dry weight in rumen contents was 2.17% (w/v). The dry matter in the rumen fluid supernatant was 1.33% (w/v); its N content was 0.41% (w/w dry matter). The measured dry matter in the total rumen contents was 12.38%, standard deviation 1.27% (w/v). This parameter, measured 21 times over a 5-month period, showed no consistent tendency to change. With 2.17 and 1.33% dry matter in the microbial cells and the dissolved solids, the plant particulate material amounts to 8.88% (w/v). If it totally excluded PEG, the estimated rumen volume is wrong by this percentage amount; the error is less to the extent that PEG penetrates the solids.

The samples of liquid rumen contents (LSP) removed from within the sampling tube (Table 3 and 4) differed in dry matter (4.45%, standard deviation 0.84%) from the total rumen contents (12.38%). The average percentage of N (non-ammonia) in the pooled dry matter of the sampled LSP material was 3.855% as compared to 2.445% for the total rumen contents.

In Table 6 are shown the *p*H and the concentration of volatile fatty acids and ammonia in the rumen contents. The *p*H did not vary much within the 2-hr feeding period. The other values show a coefficient of variation of about 10%.

Three in vitro measurements with whole rumen contents of the rate of carbon dioxide production from organic carbon gave values of 2.36, 2.36, and 2.62 mmoles per 100 g per hr, an average of 2.45, or 2.76 moles/day for the 4.7 liters of rumen contents.

Fifteen similar measurements of the rate of methane production gave values ranging from 0.51 to 1.01, for an average of 0.74 mmoles per 100 g per hr or 0.83 moles/day in the entire rumen

Zero-time rate values for production of ammonia in incubated whole rumen contents were 0.44, 0.54, 0.30, and 0.50, for an average of 0.445 mmoles per 100 g per hr, equal to 0.50 moles of ammonia per rumen per day.

The rates of formation of the volatile fatty acids are shown in Table 7.

Initially, the proximate analyses of feed and feces (Table 1), with assumed chemical composition for each component, were used in chemical equations to describe the fate of material disappearing during passage of feed through the sheep on the 2-hr regime. The summed values represented the estimated feed and fecal composition. The large amount of "other carbohydrates," determined by difference, was of unknown composition. Further, this method assumed elemental compositions for each component as the basis for calculating the total composition. Use of the direct elemental analysis of the total material to express the initial and final composition seemed more reliable.

The daily feed-feces difference in the amounts of each element (Table 1) have been used as the reactant ($C_{20.08}H_{36.99}O_{17.405}N_{1.345}$) in equation 2. The average rates of VFA formation (Table 7) amount to $C_{12}H_{24}O_{10.1}$ g atoms/day from the rumen.

The 12 g atoms of C in the VFA, the 2.76 in the CO_2, and the 0.83 in the methane leave 4.44 missing out of the 20.03 g atoms of C in the feed-feces difference. This C was assumed to be in the microbial cells formed. From the C and H content of the bacterial cell reported by Mayberry et al. (20) and assumed values of 9.39% N and 5.19% ash, an oxygen content of 32.22% was calculated for microbial cells. The 9.39% N value was estimated from an assumed nitrogen content of 8% for the protozoa and 10.5% for the bacteria (12), the relative quantities of each being estimated from Table 5. By dividing the percentage values of C, H, O, and N by the respective atomic weights, a proportion of $C_{3.8}H_{7.6}O_{2.01}N_{0.671}$ in

TABLE 3. *Concentration of various species of protozoa in the liquid-small particle (LSP) fraction and in the total rumen (TR) contents (2-hr feeding)*

Protozoa	Protozoal count at various sampling times (10^3/ml)																Ratio (avg TR count/ avg LSP count)	Total wet wt		N content of	
	5 March 19:45		23 March 11:15	19 March 11:45	25 March 11:15		16 April 10:15		17 April 11:15		27 May 11:15		28 May 10:15		Avg			LSP (mg/ml)	TR (mg/ml)	LSP (g)	TR (g)
	LSP[a]	TR[b]	LSP	TR	LSP	TR	LSP	TR	LSP	TR	LSP	TR	LSP	TR	LSP	TR					
Entodinium caudatum	65.3	45.6	48.5	45.6	58.1	36.0	118.1	120.5	64.8	96.5	39.8	49.4	27.4	29.8	60.3	60.5	1.00	6.37	6.37		
E. vorax	1.9	9.1	4.3	3.4	3.8	5.8	6.7	5.3	1.9	1.0	2.4	4.8	2.4	1.9	3.3	4.5	1.36	1.41	1.92		
Other *Entodinium* species, chiefly *E. simplex*	276.0	272.2	327.8	437.8	371.5	527.0	548.2	551.0	441.8	441.6	315.4	428.2	225.1	296.2	353.6	422.0	1.19	17.50	20.8		
Dasytricha ruminantium	5.3	4.8	9.1	6.7	18.2	18.2	27.4	30.2	38.4	35.3	4.3	4.3	1.4	4.8	14.9	14.9	1.00	2.10	2.10		
Isotricha prostoma and *I. intestinalis*	13.4	8.2	3.8	3.4	15.9	23.5	34.6	32.2	13.4	13.4	8.6	16.3	6.7	15.7	13.5	16.0	1.19	28.22	33.6		
Eudiplodinium affine	1.0	1.0	5.8	29.8	4.3	12.0	18.2	23.5	12.9	14.4	7.2	21.1	1.9	7.2	7.3	15.6	2.14	4.72	10.1		
Ostracodinium triloricatum	37.9	55.7	17.3	37.9	24.5	50.4	30.7	66.7	50.4	77.3	19.7	61.4	12.0	33.1	27.5	54.6	1.99	17.79	35.4		
Polyplastron multivesiculatum	7.7	6.2	4.3	11.5	6.2	5.8	1.9	11.0	7.2	9.1	3.8	8.2	2.9	3.8	4.8	7.9	1.65	11.62	19.2		
Ophryoscolex caudatus	16.3	18.2	9.1	35.5	15.4	28.3	18.7	42.2	22.1	25.9	6.2	34.6	1.9	15.4	12.8	28.3	2.21	25.34	56.0		
Total	424.8	421.0	430.0	611.6	515.9	707.0	804.5	882.6	623.4	714.7	407.4	628.3	281.7	407.6	498.0	624.3		115.1	185.5	3.47	6.32

[a] Count was made on a sample of the LSP passing through the perforations in the sampling tube.
[b] Count was made on a sample of the TR contents.

TABLE 4. *Concentration of various types and sizes of bacteria in the liquid-small particle (LSP) fraction and in the total rumen (TR) contents (2-hr feeding)*

Bacteria	Bacterial count at various sampling times (10^7/ml)														Total wet wt		N content	
	23 March 11:15	19 March 11:15	25 March 11:15		16 April 10:15		17 April 11:15		27 May 11:15		28 May 10:15		Avg		LSP (mg/ml)	TR (mg/ml)	LSP (g)	TR (g)
	LSP	TR	LSP	TR	LSP	TR	LSP	TR	LSP	TR	LSP	TR	LSP	TR				
Chains of cells (0.8–1.0 μm)	28.5	30.6	16.8	25.3	26.5	33.6	14.2	8.4	16.8	29.5	33.6	45.2	22.7	28.8	1.25	1.59		
Cocci and short rods (0.5–1.0 μm)	2,016	2,890	2,184	3,594	2,960	2,580	1,176	2,824	1,848	2,520	1,680	2,016	1,977	2,737	21.8	30.1		
Selenomonas and Quin's ovals (2.4 × 3–2.4 × 8 μm)	143	84	85	85	320	168	140	48	50	48	25	34	127	78	75.4	46.3		
Rods and small ovals (2–5 μm)	168	120	84	28	33.6	50.1	28.3	11.7	30.2	168	50.4	29.5	65.7	67.9	2.17	2.24		
Sarcinae (3 × 3 μm)	6.3	5.2	13.4	3.4	1.7	1.7	1.0	1.0	3.4	3.4	8.4	3.4	5.7	3.0	1.69	0.9		
Total	2,362	3,129	2,383	3,736	3,382	2,833	1,390	2,893	1,948	2,769	1,797	2,128	2,198	2,748	102.3	81.13[a]	4.54	3.81

[a] Because large particles, containing bacteria, were removed from the sample of whole contents, the count on LSP is regarded as a more reliable measure for whole contents and has been used to represent both the LSP count and the TR contents count.

TABLE 5. *Average microbial volume, numbers, and weight*

Microbe	Vol/cell (μm^3)	Determination at 2-hr feeding			Determination at 12-hr feeding	
		No.[a]	Vol (μliter)	Weight (mg/ml)	No.[a]	Weight (mg/ml)
Types of bacteria						
Chains 0.8–1.0 × variable length	5	22.7	1.14	1.25	105.1	5.78
Selenomonas and Quin's ovals 2–4 × 5–8 μm	54	127.0	68.58	75.44	27.3	16.22
Rods and small ovals 1 × 2–5 μm	3	65.7	1.97	2.17	79.2	2.61
Cocci and short rods 0.6 × 1.2 μm	1	1,977.0	19.77	21.75	2,859.9	31.45
Sarcinae 3 × 3 μm	27	5.7	1.54	1.69	1.8	0.53
Total bacteria per ml		2,198.1	93	102.30	3,072.4	56.59
Species of protozoa						
Entodinium sp. chiefly *E. simplex*	45	353.6	15.91	17.50	184.2	9.12
E. caudatum	96	60.3	5.79	6.37	25.9	2.72
E. vorax	387	3.3	1.28	1.41	0.8	0.34
Dasytricha	128	14.9	1.91	2.10	3.8	0.54
Isotricha	1,900	13.5	25.65	28.22	3.0	6.27
Eudiplodinium affine	588	7.3	4.29	4.72	3.9	2.52
Ostracodinium	588	27.5	16.17	17.79	5.8	3.75
Polyplastron	2,200	4.8	16.56	11.62	2.7	6.53
Ophryoscolex	1,800	12.8	23.04	25.34	6.8	13.46
Total protozoa per ml		498.0	104.60	115.07	236.9	45.27
All microbes, total per ml				217.37		101.86

[a] Numbers of bacteria expressed as 10^7 per milliliter; protozoa, 10^3 per milliliter.

TABLE 6. *Results of analyses of rumen contents*

Determination	Time	Amt (mmoles) of acid/100 g of rumen contents				NH_3 (mmoles/100 g)	pH
		Acetate	Propionate	Butyrate	5 Carbon		
Date							
28 May 1969	8:45	5.39	1.49	0.73	0.20	1.30	6.4
	9:15	5.66	1.82	1.11	0.18	1.16	6.5
	9:45	5.65	1.74	1.00	0.22	0.97	6.6
	10:15	4.30	1.85	0.98	0.22	1.43	6.2
27 May 1969	9:45	5.72	1.86	0.78	0.19	1.03	6.6
	10:15	5.39	2.00	0.89	0.22	1.55	6.4
	10:45	4.92	2.09	0.97	0.24	1.36	6.4
	11:15	5.00	1.89	0.96	0.22	1.24	6.3
Analysis							
Mean $\bar{X}$		5.25	1.84	0.93	0.21	1.255	6.43
Standard deviation		0.487	0.179	0.124	0.023	0.186	0.13
Coefficient of variation		9.3	9.7	13.3	10.5	14.8	2.02

microbial cells was obtained, representing 94.81% of the dry weight. This was corrected to 4.44 mg atoms of C, giving $C_{4.44}H_{8.88}O_{2.35}N_{0.785}$ as the amount of these elements in microbial cells.

The equation including the missing C as microbial cells and measured values for the daily disappearance of feed and appearance of fermentation products is

TABLE 7. *Rates of production of volatile fatty acids*

Date	Time	Initial concn of volatile fatty acids (mmoles/100 g)	Rates of production				
			C_2 (mmoles /100 g hr)	C_3 (mmoles/ 100 g hr)	C_4 (mmoles/ 100 g hr)	C_5 (mmoles/ 100 g hr)	Total (mmoles/ 100 g hr)
25 February 1969	10:15	11.38	2.50	0.57	0.30	0.06	3.43
5 March 1969	9:45	11.37	2.85	0.53	0.24	0.06	3.68
4 July 1969	11:00	11.45	3.10	1.30	0.40	0.10	4.90
27 May 1969	11:15	8.23	4.50	0.78	0.40	0.12	5.80
Avg			3.24	0.80	0.34	0.09	4.47
Avg moles per day in the rumen			3.65	0.905	0.38	0.10	4.94

TABLE 8. *Recovery of elements in the feed-feces difference*

Material	Amt of element (g atoms)[a]			
	Carbon	Hydrogen	Oxygen	Nitrogen
Feed-feces difference	20.03	36.99	17.405	1.345
Water		1.13	0.565	
(Total)	(20.03)	(38.12)	(17.970)	(1.345)
Products				
VFA	12.0	24.0	10.1	
CH_4	0.83	3.32		
CO_2	2.76		5.52	
NH_3		1.50		.50
Ash-free cells	4.44	8.88	2.35	0.785
(Total)	(20.03)	(37.7)	(17.97)	(1.285)
Per cent recovery	100 (arbitrary)	98.6	100 (arbitrary)	95.5

[a] Values for feed-feces difference and water indicate amounts initially present; values for products represent amounts recovered.

$$C_{20.03}H_{36.99}O_{17.405}N_{1.345} + 5.65\ H_2O \rightarrow C_{12}H_{24}O_{10.1} + 0.83\ CH_4 + 2.76\ CO_2 + \text{fatty acids}$$

$$0.50\ NH_3 + C_{4.44}H_{8.88}O_{2.35}N_{0.785}\ \text{microbial cells absorbed} \quad (2)$$

The balance of H and N in this equation to account for the feed-feces difference is shown in Table 8.

In equation 2 the ash-free microbial cell dry weight is 110.5 g/day or 116.5 g including ash, containing 11.0 g of N. This represents the cells digested and absorbed. Any undigested and unabsorbed microbial material would not be detected as feed-feces difference. If it is assumed that the feed nitrogen is totally converted into either microbial cells or ammonia, the 4.69 g of N in the feces represents the microbial cell N produced but not absorbed. Addition to the 11 g of microbial N of equation 2 gives 15.69 g of total microbial N formed, corresponding to 167 g (dry weight) of cells per day, with a digestibility of 69%. The microbial N leaving the rumen at the PEG rate is 15.69 g per day, or 6.9 g per turnover. The dissolved nonammonia N in the entire rumen was 0.20 g.

Of the 4.7 liters of total rumen contents, 582 g (12.38%) was dry matter containing 14.2 g of N (2.445%), of which 14.0 g was not dissolved and was assumed to be chiefly microbial. With 6.9 g of microbial N and 0.2 g of dissolved N leaving the pool with PEG, 7.1 g of N remained in the rumen as part of the "rumination pool" (13). From the direct count estimates, the 185.5 mg of protozoa per ml of rumen contents (Table 3) and the 102.3 mg of bacteria (Table 4) amount to 6.97 g of protozoal N and 5.06 g of bacterial N, or a total of 12.03 g of microbial N in the entire rumen, of which 5.13 constituted a rumination

pool. Both methods of calculation indicate a sizable retention of microbes in a rumination pool.

The values for dry matter also indicate a rumination pool of considerable magnitude. The dry matter leaving daily includes the 369 g recovered as feces, the 116.5 g of microbial cell material not recovered in the feces (equation 2), the 57 g of VFA absorbed (calculated from Table 6), and the 43 g of minerals not recovered in the feces (Table 1), for a total of 586 g per day. With 582 g of dry matter in the entire rumen contents, a turnover rate of only once per day would supply the 586 g leaving daily. Such a rate indicates that the total rumen dry matter cannot turn over at the rate (2.27 turnovers per day) found with PEG. On the assumption that the only way dry matter and microbes leave the rumen is with the PEG pool, the amount in the rumen at any one time, leaving with PEG, is 586/2.27, or 258 g. The dry matter (324 g) remaining in the rumination pool does not leave with PEG, but through comminution during digestion, mixing, and rumination continuously contributes small particles to the pool leaving with PEG. This dry matter contains 7.3 g of N (2.25%) as compared to 2.67% in the dry matter leaving with PEG.

The 6.9 g of microbial N leaving with PEG, with a volume of 679 ml, makes the volume of material leaving with PEG a total of 4.44 liters. The 258 g of dry matter in this volume gives a dry matter of 5.84% (w/v).

In Table 9, the results of experiments with the 2-hr and the 12-hr feeding (Table 5) are collected. Comparisons show that under the 12-hr feeding regime the rumen volume was larger, its dry matter less, and the turnover slower. Also, the concentration of both bacterial and protozoal cells was smaller. The estimated total microbial N supplied under the 2-hr feeding system is almost double that with the feed given twice daily.

TABLE 9. *Comparison of the 2-hr and 12-hr feeding systems*

Determination	2 hr	12 hr
Polyethylene glycol turnover rate constant	2.27 per day	1.46 per day
Rumen volume, from polyethylene glycol	4.7 liters	6.320 liters
Dry matter in rumen	0.582 kg	0.461 kg
Estimated protozoal nitrogen leaving with LSP[a]	2.12 g[b]	2.13 g[c]
Estimated bacterial nitrogen leaving with LSP	4.54 g	3.57 g[c]
Turnover rate × protozoal nitrogen in LSP	4.82 g	3.11 g
Turnover rate × bacterial nitrogen in LSP	10.87 g	5.12 g
Total microbial nitrogen leaving rumen	15.79 g	8.32 g

[a] LSP, liquid-small particle fraction.
[b] Rest of the protozoa assumed to be in the rumination pool.
[c] Includes the total nitrogen in the rumen.

DISCUSSION

Most of the values measured in the sheep are similar in magnitude to those reported by others. Rumen fluid turnover rate constants and rumen fluid volume for the 2-hr and 12-hr feeding agree with the estimates of Hogan et al. (8) and Hyden (17), respectively. VFA and ammonia-N concentrations are in good agreement with the estimates in the literature (5, 8, 23). The zero-time rates of VFA production are similar to the results obtained with a radiotracer technique in sheep fed continuously with approximately the same daily amounts of feed (6, 8, 23). The ammonia production estimates are within the ranges reported by Pilgrim et al. (23). Hungate (13) reported CH_4/CO_2 ratios of 0.22 to 0.50 for various grazing animals as compared to 0.30 in the present trial. Protozoal N yield per day for the 12-hr feeding experiment agrees with the estimate of Reichl (26) for similar feeding conditions. Weller et al. (28) found in sheep fed once daily that 50 to 82% of the total nitrogen in the feed was assimilated into microbial cells. The value in our experiments is 83%.

The 2-hr and 12-hr comparisons in Table 9 provide only a rough approximation of the effect of frequent feeding; the retention of microbes in the rumination pool of the 12-hr system cannot be estimated from the available data. But the results do indicate a significant superiority of frequent feeding for production of a large microbial cell crop.

There are some discrepancies in the nitrogen assumptions, calculations, and measurements. It was assumed that all feed N was converted either to ammonia or microbial cells. The method for measuring the N in rumen dry matter did not include ammonia, yet the nonammonia N by analy-

sis was 2.445% or a total of 14.2 g in the estimated total volume (4.7 liters) of the rumen. Yet, by estimates from direct counts, the rumen contained only 12.05 g of microbial N. Only 0.20 g of this discrepancy can be accounted for as dissolved N.

The 15.69 g total microbial N formed and the 7 g N in the ammonia produced amount to a total of 22.69 g of N per day as compared to the 18.83 g per day in the feed-feces difference. This increase (3.86 g) over that in the feed can be due to production of ammonia in the rumen from sources other than feed nitrogen. Urea N entering the rumen in the saliva is converted to ammonia. The total liquid volume leaving the rumen at the PEG rate is 2.27 × 4.44 liters, or 10.06 liters per day. This is approximately the volume of saliva expected per day. If all of the nitrogen (chiefly urea) in the saliva (17.6 meq/liter) (18) is converted to ammonia in the rumen, it amounts to 2.4 g per day. In addition, the urea N diffusing into the rumen from the blood can supply as much as 7.5 g of N per day to the rumen (9). The total possible rumen N supplied from all of these sources amounts to 28.73 g. Since 7 g appeared as ammonia, a maximum of 21.73 could theoretically have been assimilated into microbial cells, as compared to the 15.69 g of equation 2.

If this additional N were synthesized into microbial cells, absorbed, and metabolized by the host, the N recycled from saliva and blood would not appear in equation 2, being assimilated into the host or excreted as urea. Thus the N balance in equation 2 is not a check on microbial N assimilation. But in such additional microbial cells, also, the H, derived from the feed, would not be recovered. The good H balance in equation 2 is not consistent with extensive microbial assimilation from recycled N.

The balance for H atoms in Table 8 is much better than expected, considering the errors and estimates involved, particularly since any errors in C and O would be reflected in a discrepancy in H.

The evidence for such a large rumination pool in this animal was unexpected. The rumen contents seemed fairly homogeneous when removed with the large-bore glass tube and the particles were small as compared to those in animals consuming hay. But the evidence from the direct estimate of microbial N by microscopic examination, as well as from measured total N in the rumen both indicate that the rumen nitrogen (chiefly microbial) did not turn over at the PEG rate.

The rumen dry matter content also cannot turn over at the PEG rate and at the same time give only the amount of dry matter recovered in the feces. Digestive activity posterior to the forestomach could hardly account for the disappearance of the 324 g of dry matter in the rumination pool and disappearance of this amount would be inconsistent with the feed-feces difference.

If all of the rumen microbial N (12.05 g) turned over with PEG, a total of 27.35 g per day would leave the rumen. This excess over the 15.69 g of microbial N produced per day indicates that the net average rate of passage or turnover rate of the microbes is 15.69/27.35 × the PEG rate, or 1.25 per day.

It is doubtful that this average passage rate. for microbial N applies equally to all species Some bacteria such as *Bacteroides succinogenes*, adhering to plant particles, may be delayed in passage more than loose-floating cells such as *Streptococcus bovis*.

The slower rate for microbial turnover as compared to PEG can account for a discrepancy between measured turnover rates of the rumen and the rates of growth observed in vitro for some of the large rumen protozoa. *Polyplastron multivesiculatum* (10; Coleman, Proc. Soc. Gen. Microbiol., vol. 61, p. iv., 1970) and *Ophryoscolex purkynei* (19) do not divide in vitro more often than once per day. Division each 7.3 hr is required to maintain their concentration in a continuous system turning over 2.27 times per day. But if, due to their high specific gravity or to a tendency to remain with the particles not passing with PEG, their average passage rate was only 0.69 per day, a division each 24 hr would be sufficient to maintain their numbers in the rumen.

Inspection of Table 3 shows that the concentrations of *Ophryoscolex*, *Polyplastron*, *Ostracodinium*, and *Eudiplodinium* were considerably greater in whole rumen contents than in the LSP pool sampled. This is consistent with a turnover rate slower than PEG. Since these large protozoa contain about 65% of the total protozoal nitrogen, such retention could be sufficient in magnitude to account for a considerable part of the slow rumen microbial passage as compared to the LSP pool.

For rapid and complete fermentation of food and production of a maximal microbial crop within a limited time, retention of microbes in a rumination pool has the same advantages as cell feedback in a continuous culture (24). A larger population of microbes is retained in the rumen than could be held there if the total rumen contents turned over at the PEG rate. This may be an important factor in the success of the rumen fermentation of fiber. It may also explain the preponderance of starch-digesting protozoa in ruminants well adapted to a grain ration. The retained population of protozoa is so large that it

ingests and thereby sequesters from *S. bovis* and other amylolytic bacteria much of the starch consumed; the bacteria cannot grow explosively as they do in hay-fed animals suddenly given grain. The latter do not contain the high concentration of protozoa (14).

In batch culture, Bauchop and Elsden (1) found cell yields of *Streptococcus faecalis* to be 19 to 23 g/mole of glucose or 11.7 to 14 g/100 g of glucose added. Hungate (12) in continuous cultures of *Ruminococcus albus* found an average dry cell yield of 26.3 g/100 g of cellobiose used. Hobson and Summers (7) obtained values as high as 47 g/100 g. The dry cell yield for the 2-hr system is 27.3 g/100 g of feed-feces difference. This agrees with the estimates of Hume (9) for sheep on a 3-hr feeding interval.

If all of the 20.03 g atoms of C in the feed-feces difference is assumed to be hexose (3.34 moles), the yield of microbial cells for the 2-hr feeding is 50 g of microbes/mole of hexose. This is near the top of the values reported by Payne (22).

The 1.345 g atoms of N in the feed-feces difference indicates that about 5 g atoms of the feed C was in protein (assumed to contain 16% N and 52.5% C). This leaves 15 g atoms of C in hexose, equivalent to 2.5 moles. The cell yield then becomes 67 g/mole of hexose used, equivalent to 6 adenosine triphosphate (ATP) molecules per mole of hexose, if 11 mg cells are derived from one ATP molecule, the value indicated in the extensive review by Payne (22). Two further factors influence this figure: (i) the actual value is less because the protein was fermented along with the carbohydrate, although less efficiently for cell production, and (ii) the actual value may be more because part of the feed carbohydrate is assimilated into cell material, diminishing the extent to which it can supply ATP.

A high ATP yield may result from the greater number of ATP-yielding reactions possible in mixed as compared to pure cultures.

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