Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system

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1. A procedure for sampling digesta from within the omasal canal of sheep given a variety of roughage diets was used to enable comparison to be made of the composition of effluent from the reticulo-rumen with that of rumen fluid.

2. Concentrations of protozoa in effluents, relative to a soluble marker continuously infused intraruminally, were usually less than 20% of corresponding rumen fluid concentrations. It was estimated that the amount of protozoal nitrogen leaving the rumen represented less than 2% of dietary N.

3. Passage of volatile fatty acids (VFA) from the rumen in effluent was less than 75% of that indicated by rumen concentrations.

4. A continuous, in vitro fermentation system was developed, in which outputs of protozoa were comparable with in vivo outputs.

Assessments of the nutritional significance of protozoa and other components of digesta passing from the rumen have often been based on concentrations measured in the rumen. In the present work a more direct approach was made by attempting to collect digesta emerging from the reticulo-omasal orifice into the omasal canal. Examination of this material indicated passage of protozoa that was much lower than would have been calculated from rumen fluid concentrations and flow rates of liquid to the omasum, suggesting a considerable degree of sequestration of these organisms within the rumen.

In the development of continuous, in vitro fermentation systems designed to simulate rumen conditions, the maintenance of appropriate concentrations of protozoa was of considerable importance. Until recently (Abe & Kumeno, 1973) success has been very limited, and in view of the present indication of sequestration within the rumen, it is likely that this success may depend upon providing in vitro conditions under which a similar degree of sequestration is possible. An aim of the present work was to develop an in vitro system capable of maintaining an output of protozoa comparable with the output measured in rumen effluent.

EXPERIMENTAL

In vivo trials

Two Merino ewes were fitted with fistulas in both rumen and omasum, the latter by the procedure of Willes & Mendel (1964) as modified by Hume, Moir & Somers (1970). The cannula fitted to the omasum was moulded from 'Corvic' paste (P 65–60; ICI Fibres Ltd, London SW 1) with an external diameter of 34 mm and an inner flange lanceolate in shape to align with the omasal laminas and provide a leak-free

seal. Portions of a few laminas were excised to facilitate access to the reticulo-omasal orifice.

The sheep were fed automatically, at hourly intervals, with equal portions of the daily rations shown in Table 1, and water was available at all times. A preliminary stabilizing period of at least 16 d was allowed with each dietary regimen. Throughout each trial period of 7 d a solution containing sodium [1-^{14}C]acetate and the complex of chromium with ethylenediaminetetraacetic acid (Cr EDTA) was continuously infused intraruminally at a rate of 0·5 l/d to provide 0·2 g Cr and 5 μCi ^{14}C daily.

Samples of effluent digesta from the rumen were drained from the omasal canal by inserting a tube of 20 mm internal diameter through the cannula into close proximity with the reticulo-omasal orifice and collecting in polythene bags for 1 h periods. Two such collections were made each day for the last 5 d of the trial period, and analyses performed on individual samples after squeezing through Terylene voile to remove larger particles. Midway through each effluent collection, rumen fluid was withdrawn from the mid-ventral sac using gentle suction through a probe which terminated in a stainless-steel mesh cylinder of diameter 20 mm and length 70 mm, covered with Terylene voile. The ratios, protozoa concentration: Cr concentration and volatile fatty acid (VFA) concentration:Cr concentration for each rumen fluid sample were compared with those for the corresponding effluent sample.

In three trials the rumen effluent was collected in a series of approximately 15 g fractions which were then bulked into two separate samples. The 1st sample included all fractions with pH less than that of the corresponding rumen fluid sample and the remaining fractions formed the 2nd sample.

Throughout each 5 d collection period, rumen fluid was withdrawn continuously at a rate of 40 ml/h and returned to the rumen at 33·5 ml/h, using a multi-channel roller pump. A 'T'-piece in the loop between the two pump tubes allowed collection of rumen fluid at a rate of 6·5 ml/h for use in the determination of rumen VFA production. On the 3rd day of sampling in most trials a sample of about 250 g whole rumen contents was collected and used for the isolation of washed protozoa.

In vitro trials

Concurrently with four of the in vivo trials, continuous in vitro fermentations were performed using rumen contents from the same animal as inoculum and 50 g/d of substrate of the same composition and physical form as that of the diet. The procedure was developed from that of Aafjes & Nijhof (1967) and is described in detail in the Appendix. Inoculation began 4 d before in vivo sampling, enabling the fermentations to reach and maintain stability throughout the following 5 d collection of both in vitro and in vivo samples. Fermentations were conducted in quadruplicate and daily outputs of protozoa and VFA measured in the effluents.

Methods of analysis

The concentrations of protozoa were measured using a Sedgewick Rafter counting chamber. Individual species were not identified but a note was taken of the proportion of holotrichs in the total population, in which small entodiniomorphs predominated

in every instance. Preparations of protozoa relatively free from plant fragments and bacteria were made by straining rumen contents through Terylene voile, centrifuging at 170 *g* for 15 s and washing twice with water. Nitrogen in washed protozoa was determined by the Kjeldahl method using a portion of a suspension containing a known number of protozoa.

Cr was determined by atomic absorption spectrophotometry and chloride with an automatic titrator (Buchler-Cotlove, Chloridometer). VFA and ^{14}C in VFA were measured as previously reported (Weller, Gray, Pilgrim & Jones, 1967).

RESULTS

Passage of protozoa from the rumen

Concentrations of protozoa in rumen fluid and rumen effluent are shown in Table 1, together with numbers of protozoa in effluent per unit amount of marker, expressed as a percentage of the numbers in rumen fluid per unit amount of marker. In all instances means are for the ten samples taken during the last 5 d of each trial. The values indicate a passage of protozoa to the omasum only 6–29% of that which would have been expected if protozoa passed from the rumen at the same rate as fluid marker. In most trials proportions of holotrichs in effluent were similar to those in rumen fluid. Marker dilution was slightly greater in effluent, which may be related to the observation that during drinking, a portion of the water flowed from the omasal canal into the sample, apparently by-passing the rumen.

A further observation was that although the pH of effluent was usually slightly greater than that of rumen fluid, in occasional samples pH was reduced. When samples were collected in a series of fractions of about 15 g it was occasionally found that a small number of these fractions contained digesta with pH as low as 2·5 and chloride concentrations as high as 120 mmol/l, presumably the result of backflow from the abomasum. This phenomenon usually occurred to some extent once or twice during each hour of effluent collection and was associated with increased flow from the sampling tube. To assess the likely effect of such backflow on the composition of rumen effluent samples, fractions of digesta with pH less than that of the rumen were bulked separately from the remaining effluent in trials 4, 7 and 8 (see Table 1). The results in Table 1 show that in these three trials the inclusion of the low-pH portion of the sample with the true rumen effluent had little effect on the values for extent of protozoa passage.

Passage of protozoal N

The N content was determined for a counted number of protozoa in nine trials allowing calculation of output from the rumen of protozoal N on the basis of concentration of organisms in rumen effluent, and fluid flow rate as calculated from marker dilution. Population distributions in the washed suspensions appeared to be similar to those in the rumen effluents, but because of obvious contamination of some of the preparations with plant fragments, the values for N content of protozoa were over-estimated to varying degrees. The results in Table 2 show that protozoal N leaving the rumen amounted to 2% or less of dietary N intake.

Table 1. *Content of protozoa, volatile fatty acids (VFA) and marker chromium in rumen fluid (RF) and rumen effluent (RE) of sheep*

(Mean values; ranges in parentheses)

Trial no.	Sheep no.	Ration (g/d)	Sample	Dilution ratio, Cr in RF: Cr in RE	Protozoa: Total (no./ml (× 10^{-5})) Range	Protozoa: Total (no./ml (× 10^{-5})) Mean	Protozoa: Holotrichs (% of total)	Protozoa: $\frac{\text{Total} \div \text{Cr in RE}}{\text{Total} \div \text{Cr in RF}} \times 100$ Range	Protozoa: $\frac{\text{Total} \div \text{Cr in RE}}{\text{Total} \div \text{Cr in RF}} \times 100$ Mean	$\frac{\text{VFA} \div \text{Cr in RE}}{\text{VFA} \div \text{Cr in RF}} \times 100$ Range	$\frac{\text{VFA} \div \text{Cr in RE}}{\text{VFA} \div \text{Cr in RF}} \times 100$ Mean
1	1	WC 600, LC 200	RF	1·07	(1·5–3·9)	2·3	8	(7–22)	12	(48–74)	59
			RE		(0·12–0·41)	0·26	9				
2	1		RF	1·08	(1·4–3·8)	2·5	8	(7–18)	10	(44–69)	59
			RE		(0·14–0·30)	0·24	9				
3	1		RF	1·05	(1·6–3·2)	2·2	10	(7–20)	12	(44–65)	56
			RE		(0·10–0·52)	0·25	10				
4	2		RF		(1·6–2·3)	1·9	13				
			RE*	1·21	(0·24–0·64)	0·45	4	(15–43)	29	(61–82)	73
			RE†	1·31	(0·20–0·62)	0·40	5	(14–48)	28	(53–76)	66
5	1	WC 544, LC 184, WG 72	RF		(1·6–3·2)	2·4	7				
			RE	1·19	(0·22–0·49)	0·36	8	(13–21)	18	(55–82)	71
6	1		RF		(1·2–2·6)	1·9	8	(10–21)	14	(52–66)	61
			RE	1·14	(0·12–0·34)	0·24	6				
7	1	LC 800	RF		(3·1–4·9)	3·8	7				
			RE*	0·96	(0·14–0·42)	0·23	4	(2·7–9·9)	6	(63–72)	68
			RE†	1·00	(0·13–0·39)	0·22	4	(2·7–9·5)	6	(59–70)	65
8	2		RF		(1·3–2·0)	1·6	12				
			RE*	1·24	(0·07–0·24)	0·16	7	(7–17)	12	(64–83)	75
			RE†	1·39	(0·06–0·23)	0·14	8	(7–17)	12	(56–81)	71
9	1	LP 800	RF	1·12	(1·0–1·9)	1·4	9	(6–13)	9	(57–86)	68
			RE		(0·08–0·17)	0·11	4				
10	2		RF	1·31	(1·0–2·3)	1·8	5	(7–26)	13	(61–82)	72
			RE		(0·10–0·28)	0·18	5				

WC, wheaten-hay chaff; LC, lucerne-hay chaff; WG, crushed wheat grain; LP, ground and pelleted lucerne hay.
* Sample fraction with pH ≥ rumen pH.
† Total sample.

Table 2. *Rates of passage of protozoal nitrogen from the rumen of sheep*

Trial no.*	Rumen effluent flow† (l/d)	Protozoa in rumen effluent (no./d($\times 10^{-8}$))	N in protozoa (mg/10^8)	Protozoal N in rumen effluent	
				(mg/d)	(% of dietary N)
1	12·6	3·3	0·80	260	1·9
2	12·7	3·0	0·60	180	1·3
3	9·9	2·5	0·83	210	1·4
5	12·8	4·6	0·63	290	2·1
6	10·9	2·6	1·7	440	2·0
7	8·2	1·8	0·85	180	0·7
8	10·5	1·5	1·0	150	0·7
9	10·6	1·1	1·3	140	0·7
10	9·9	1·8	0·95	230	0·8

* For details, see Table 1.
† Calculated from marker dilution.

Table 3. *Volatile fatty acid (VFA) composition and ^{14}C activity in rumen fluid and effluent of sheep*

Trial no.*	Sample	VFA composition (mol/100 mol)			^{14}C in total VFA (μCi/mol)
		Acetic	Propionic	Butyric†	
5	Rumen fluid	68	16	16	1·08
	Rumen effluent	71	16	13	1·02
7	Rumen fluid	70	17	13	1·27
	Rumen effluent	71	16	13	1·30

* For details, see Table 1.
† Including higher-molecular-weight VFA.

Passage of VFA

In Table 1, VFA concentrations relative to Cr in rumen effluent are shown as percentages of the corresponding rumen fluid values. The results indicate that passage of VFA from the rumen with digesta ranged from 56% to 75% of amounts calculated from rumen fluid concentrations and flow rates.

Table 3 shows the compositions of VFA in bulked rumen effluent samples from trials 5 and 7 (see Table 1) differing only slightly from those in rumen fluid collected continuously throughout the 5 d. Differences in concentration of ^{14}C in total VFA were also small.

Comparison of in vitro and in vivo effluent products

Continuous in vitro fermentations were established concurrently with trials 3, 5, 6 and 9 (see Table 1) and Table 4 shows the concentrations and amounts of protozoa and VFA in effluent. The values are means of daily estimations from four replicate fermentations over 5 d. In each trial the twenty individual VFA production measurements showed little variation, but coefficients of variation in protozoa output values ranged from 18% to 26%. A comparison of products from the in vitro system with those from the rumen on the basis of intake of dry substrate or food is shown in Table 5. Although protozoa outputs were measured in effluent in both systems this was not possible with VFA as a major portion is absorbed through the rumen wall. The in vivo

Table 4. *Concentrations and amounts of protozoa and volatile fatty acid (VFA) in effluent from in vitro fermentations*

Trial no.*	Substrate (g/d)		Effluent flow (l/d)	Protozoa in effluent (no./ml ($\times 10^{-3}$))	Protozoa in effluent (no./d ($\times 10^{-6}$))	VFA in effluent (mmol/l)	VFA in effluent (mmol/d)
3	WC	37·5					
	LC	12·5	1·73	13	22	91	158
5	WC	34·0					
	LC	11·5	1·75	23	40	87	152
	WG	4·5					
6	LC	50	1·75	3·5	6·1	101	177
9	LP	50	1·73	5·7	9·9	111	192

WC, wheaten hay chaff; LC, lucerne hay chaff; WG, crushed wheat grain; LP, ground and pelleted lucerne hay.

* For details, see Table 1.

values represent total rumen VFA production measured by isotope dilution and are therefore not strictly comparable with the in vitro output measured direct in effluent.

At the conclusion of in vitro trial 9 (see Table 1) examination of the distribution of protozoa within the fermentation chambers was attempted. Although concentrations in the free fluid were about 6×10^3/ml, similar to those in effluent, fluid squeezed from the bags of solid digesta residues ranged from $7{\cdot}0 \times 10^4$/ml to $2{\cdot}3 \times 10^5$/ml. It is possible that higher protozoa concentrations in effluent would have resulted if the movement of fluid through digesta solids had been increased by using a higher rate of mechanical agitation.

DISCUSSION

The turnover rate of fluid in the rumen is markedly greater than that of digesta solids (Weller, Pilgrim & Gray, 1962, 1971). Consequently estimates of passage of protozoa from the rumen based on rumen fluid concentrations depend on the extent to which movement of these organisms is associated with fluid flow. On the assumption that rates of removal of protozoa, bacteria and fluid are equal it has been calculated that protozoal N may account for about 20% of microbial N leaving the rumen with roughage-fed sheep (Pilgrim, Gray, Weller & Belling, 1970). Support for the assumption is provided by the ease with which protozoa are separated from digesta solids. When rumen contents are strained through muslin the concentration of protozoa in the filtrate is not measurably less than in the residual fluid and it was concluded by Warner (1966) that sequestration of organisms among food particles was of major importance only with small bacteria.

Hungate (1966), on the other hand, speculated that some protozoa may maintain their numbers in the rumen by collecting around the slower-moving components of digesta. More recently Abe & Kumeno (1973) studied effects of fluid turnover on protozoa populations in vitro. They pointed out that in the rumen, protozoa concentrations were maintained despite a much greater fluid turnover than in their in

Table 5. *Amounts and compositions of protozoa and volatile fatty acids (VFA) in effluent after in vivo and in vitro rumen fermentation*

Trial no.*	Dry matter intake (g/d)	Protozoa: Effluent concentration (total/ml ($\times 10^{-4}$))	Protozoa: Holotrichs (% of total)	Protozoa: Production (total/kg dry matter intake ($\times 10^{-8}$))	VFA: Composition (mol/100 mol) Acetic	Propionic	Butyric†	VFA: Production (mol/kg dry matter intake)
3 in vivo	696	2·5	10	3·6	62	22	16	5·7
in vitro	43·5	1·3	12	5·1	55	16	19	3·6
5 in vivo	728	3·6	7	6·3	68	16	16	4·9
in vitro	45·5	2·3	2	8·8	56	19	25	3·3
6 in vivo	706	2·2	8	3·4	70	17	13	6·0
in vitro	44·1	0·4	25	1·4	60	19	21	4·0
9 in vivo	718	1·1	9	1·6	71	17	12	5·6
in vitro	44·9	0·6	1	2·2	62	18	20	4·3

* For details, see Table 1.
† Including higher-molecular-weight VFA.

vitro systems, and suggested that this may be due to a lower rate of removal of protozoa than of fluid. It was noted in some early experiments (Weller & Gray, 1954) that concentrations of protozoa in the omasal fluid of slaughtered sheep were much lower than in the rumen. At the time it was suggested that this was due to destruction of the organisms in the omasum, but the present findings indicate that sequestration of protozoa within the rumen is a more likely explanation.

Collection of digesta samples from within the omasal canal, close to the reticulo-omasal orifice, was done with the aim of reducing as far as possible the exposure of rumen effluent to omasal action. Visual observation of the orifice through the sample tube during collections suggested that the bulk of the rumen effluent passed directly to the sample. In every trial, protozoa concentrations in effluent were much lower than in the corresponding rumen fluid. The use of a soluble marker as a reference eliminated effects of absorption or dilution on the comparisons. As shown in Table 1, the passage of protozoa was much less than would have been estimated from rumen fluid concentrations, indicating a considerable degree of sequestration within the rumen.

Because of the greater digestibility of protozoal protein compared with that of bacteria (McNaught, Owen, Henry & Kon, 1954) and also its higher content of essential amino acids (Weller, 1957) there have been speculations that nutritional advantage to the animal may result from the synthesis and passage from the rumen of protozoal protein. The figures in Table 2, however, suggest that the contribution of protozoa would be too small to affect significantly the composition of the total protein mixture. The possibility remains that with different physical conditions in the rumen the degree of sequestration may be reduced, but apart from this it appears likely that any nutritional effects of protozoa must arise from metabolism within the rumen.

In addition to rumen effluent, samples from the omasal canal occasionally contained drinking-water which had by-passed the rumen (cf. Watson, 1944) and also abomasal digesta. The former phenomenon did not affect the conclusions, as a marker was used, while backflow from the abomasum was shown to cause only slight errors in estimates of protozoa and VFA passage. Evidence from continuous pH measurements within the omasal canal, with the cannula closed to maintain volume and pressure, indicated that backflow still occurred periodically (Weller, unpublished results). This contrasts with the finding of von Engelhardt & Ehrlein (1968), who detected no such evidence of abomasal backflow in goats.

The reduction in VFA concentrations relative to marker from rumen fluid to rumen effluent (Table 1) was greater than is usually found between rumen and reticulum, suggesting rapid absorption in the region of the reticulo-omasal orifice. As shown in Table 3, absorption of approximately 30% of total VFA caused little change in the molar proportions of individual acids. Measurements of ^{14}C concentrations in VFA of rumen fluid and effluent (Table 3) provide evidence that infused marker was adequately mixed within the rumen.

On the basis of measurements in rumen effluent the output of protozoa from the in vitro fermentation was comparable with corresponding in vivo measurements (Table 5). Although some differences were apparent in the proportion of holotrichs,

the degree of success in maintaining these organisms was greater than in previously reported in vitro systems. Production of VFA was consistently lower in vitro but it is uncertain whether this is a reflection of a reduced extent of fermentation or the different methods of measurement. As found by Abe & Kumeno (1973), butyric acid: acetic acid ratios were higher than in the rumen.

The reduced concentration of protozoa in rumen effluent compared with rumen fluid suggests that maintenance of protozoa numbers within the rumen may be largely dependent upon the rate of removal of protozoa being less than fluid turnover rate. It is likely that a similar relationship between these rates would be necessary in an in vitro system. Removal rates of protozoa were not determined in these experiments but the relatively high concentrations in the fluid within the digesta bags indicated sequestration at least qualitatively similar to that demonstrated in vivo. In experiments subsequent to those reported it was found possible, by adjusting the rate of mechanical agitation, to vary the ratio of protozoa concentrations within and outside the digesta bags, and maximum outputs were achieved when this ratio was about 7:1. A similar ratio between rumen fluid and effluent protozoa concentrations was found in most of the in vivo experiments (Table 1).

The conclusion may be drawn that a requirement for an in vitro fermentation in which rumen protozoa metabolism is simulated is the provision of physical conditions under which sequestration among particulate digesta is allowed to an extent sufficient to ensure a protozoa removal rate considerably less than that of fluid turnover.

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APPENDIX

Procedure for continuous in vitro fermentations

A simple, continuous fermentation system was developed by a series of modifications to the procedure of Aafjes & Nijhof (1967). Rumen-like fermentations could be maintained for several weeks by daily addition of roughage substrates together with a continuous supply of buffer solution. Solid digesta residues were removed daily, while soluble and suspended products were removed continuously in the effluent. Ionic concentrations, pH range and VFA concentrations were maintained within normal rumen limits by the choice of appropriate buffer composition and flow rate.

Details of a fermentation unit are illustrated in Fig. 1. Digesta solids and substrate are enclosed in Terylene mosquito-net bags (*d*) held to a cylindrical shape by stainless-steel wire formers (*e*). Two such cartridges are contained in a stainless-steel mesh basket (*a*) of diameter to provide a sliding fit within a 2 l polypropylene cylinder (*f*). A rod (*p*) is attached to the basket and passes through a Perspex lid containing openings (*h*, *g*) for infusion, sampling and pH measurement. The cylinder is fitted with an outlet tube (*k*) for effluent and a gas inlet (*m*). In the complete apparatus, four such fermentation vessels are immersed in a water-bath at 40° and each rod (*p*) is attached to one of four arms projecting radially from a vertically reciprocating shaft above the

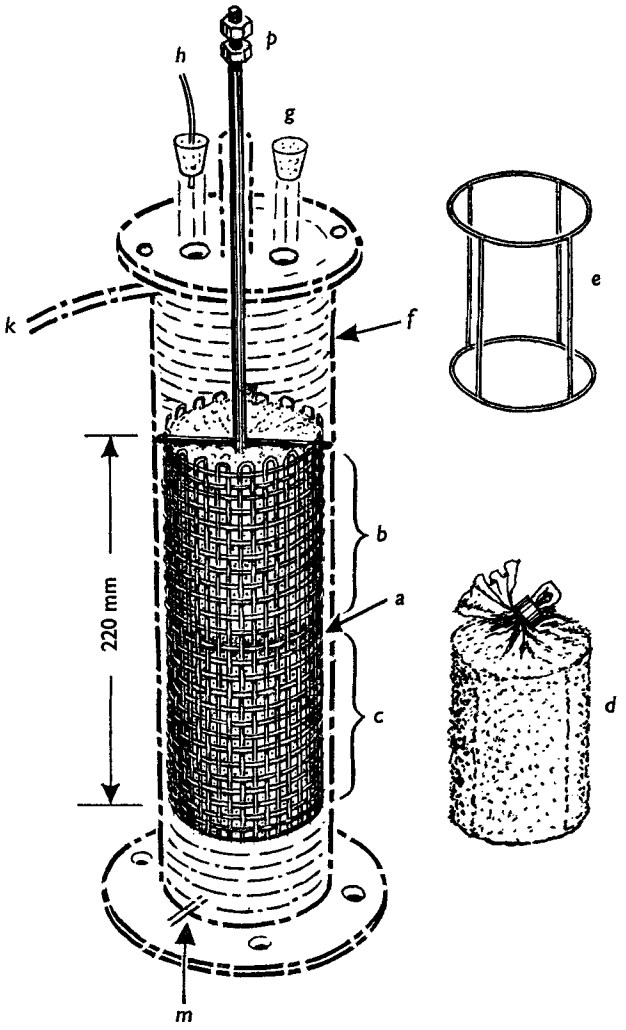

Fig. 1. In vitro fermentation vessel. *a*, steel mesh basket; *b*, *c*, positions of digesta bags; *d*, digesta-solids bag; *e*, wire former; *f*, 2 l polypropylene cylinder; *g*, *h*, inlets for sampling and infusion; *k*, effluent tube; *m*, gas inlet; *p*, driving rod.

fermentation chambers. This shaft is driven at a rate of 8 strokes/min with a stroke length of 80 mm.

Buffer solution, containing (g/l): $Na_2HPO_4.2H_2O$, 2·2; $NaHCO_3$, 5·0; KCl, 0·6; $KHCO_3$, 1·6; urea, 0·2, is infused into each vessel at a constant rate of about 1·5 l/d. A gas mixture of N_2 and CO_2 (95:5) is bubbled slowly through the inlet tubes (*m*). Effluent is allowed to flow into stirred vessels containing 30 ml formalin for each 24 h collection.

Solids from 750 g fresh rumen contents mixed with sufficient substrate to almost fill the bag (*d*) form the inoculating charge which is installed in position (*c*) and a bag containing the daily charge of 50–60 g dry weight of substrate is added in position (*b*). The fluid from the inoculum is held in the vessel (*f*). The drive mechanism, buffer

flow and gas bubbling are allowed to proceed for 24 h, after which the inoculating charge is removed, the wire former withdrawn and excess fluid squeezed back into the fermentation vessel. The initial substrate charge is lowered to position (*c*), a fresh charge inserted in position (*b*) and a further inoculum of 100 ml rumen fluid added to the vessel. Thereafter the procedure is repeated daily but without further inoculation.

The fermentation reaches equilibrium within about 4 d and may then be maintained for an indefinite period with steady outputs of VFA and protozoa. The pH of free fluid in the chamber reaches a maximum before each daily substrate addition and the supply of buffer is adjusted to ensure that this maximum is within the range 6·3–6·7. It is necessary to produce adequate movement of fluid through digesta solids by ensuring a sufficiently close fit of the basket within the cylinder and, if necessary, by adjusting the agitation rate. Satisfactory maintenance and production of protozoa is usually achieved when the concentration in fluid squeezed from the digesta bags is five- to tenfold that in the free fluid and of the same order as that found in the rumen of the donor animal.

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