Dynamics of protozoa in the rumen of sheep

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1. Protozoa were labelled by incubating 100 ml rumen fluid with [^{14}C]choline for 1 h. The protozoa were concentrated by centrifugation and then washed with rumen fluid. This reduced residual ^{14}C in the fluid medium to insignificant amounts while still retaining the viability of the labelled protozoa. Washing procedures using formal saline (40 g formaldehyde/l saline (9 g sodium chloride/l)) and saline were developed to isolate protozoa for estimation of specific radioactivity.

2. The protozoal pool in freshly-collected rumen fluid incubated in vitro retained 90% of the radioactivity for up to 6 h following addition of ^{14}C-labelled protozoa produced as indicated previously. The specific radioactivity of protozoa did not change during the incubation period.

3. Protozoa labelled with [^{14}C]choline and then stored until they died rapidly lost ^{14}C to methane when they were incubated in rumen fluid or were injected into the rumen. Some [^{14}C]choline was salvaged under these conditions by the live protozoa present as they apparently incorporated up to 13% of the label from the dead protozoa. However, protozoal debris from the injected solution could also have been present in the isolated protozoa.

4. The in vitro results suggested that the protozoal preparations were viable, and that the incorporated choline did not have a turnover in excess of the turnover of nitrogen (i.e. specific radioactivity remained constant with time in vitro) suggesting that the dilution of specific radioactivity of protozoa following mixing of a ^{14}C-labelled dose of protozoa represented the rate of irreversible loss and also replacement of protozoa in the rumen.

5. ^{14}C-labelled protozoa had a half-life in the rumen which was greater than that of rumen fluid and in six animals the protozoal replacement rate was 1–4·1 mg N/min.

6. Losses of ^{14}C from labelled protozoa in the rumen in methane or via abomasal digesta were 65 and 35% respectively.

7. The results suggest that protozoal growth may be as high as 32% of the total microbial protein synthesis in the rumen but that 65% of the protozoa die and are degraded in the rumen.

The requirement for dietary amino acids by ruminants is dependent on the efficiency of microbial cell growth which appears to be substantially below the theoretical maximum (see Hespell & Bryant, 1979; Leng, 1982). Part of the apparent inefficiency in microbial cell growth, and therefore availability of microbial protein to the animal, may be attributable to the turnover of microbial cells in the rumen (Nolan & Leng, 1972). The apparent retention of protozoa in the rumen (Weller & Pilgrim, 1974; Minor *et al.* 1977; Bird *et al.* 1978; Harrison *et al.* 1979) and the increased microbial protein flow to the duodenum on defaunation of sheep (Lindsay & Hogan, 1972), together with observation of significant quantitative engulfment of bacteria by protozoa (Coleman, 1975), all suggest that a large population of protozoa may lead to a low rate of microbial protein synthesis or flow-rate to the small intestines or both. In addition recent studies of microbial growth in ruminal digesta in vitro have indicated that the presence of protozoa increased the turnover of bacterial cells and this lowered the net yield of microbial protein (Demeyer & Van Nevel, 1979).

In cattle and sheep on high-energy diets (that are also high in non-protein-nitrogen (NPN) but low in true protein) with large populations of protozoa in the rumen, significant increases in live-weight gain have resulted where protozoa were eliminated (Bird & Leng, 1978; Bird *et al.* 1979). In sheep, defaunation has also been associated with increased wool growth (Bird *et al.* 1979; Bird & Leng, unpublished results) suggesting there was an increase in protein outflow from the rumen. These results suggest that the partitioning of end-products

of fermentation between microbial cells and volatile fatty acids (VFA), plus methane and carbon dioxide is directed more towards microbial cells in the defaunated as compared to the faunated ruminant.

There have been few quantitative studies of the growth rates of protozoa in the rumen. Most investigations have been concerned with assessing the flow of protozoal protein to the dudoenum (Harrison *et al.* 1979). In preliminary studies Coleman *et al.* (1980) used protozoa labelled with [^{14}C]choline to study their flow out of the rumen. Leng *et al.* (1981) used similar techniques to study the turnover of large protozoa in cattle on sugar-cane-based diets and showed that this was apparently very slow.

In the studies now presented the turnover of smaller protozoa (largely oligotrichs of the species *Entodinium*) in the rumen of sheep has been estimated using isotope dilution procedures. Protozoa labelled with [^{14}C]choline in vitro were introduced into the rumen of sheep and the specific radioactivity of protozoa in the rumen determined over 2–3 d. The loss of radioactivity in methane and also that flowing into the abomasum in digesta were also estimated. Although the apparent rate of protozoal protein synthesis was high relative to that in the previous studies (Leng *et al.* 1981), approximately one-third of the labelled protozoa flowed to the abomasum in digesta. Approximately 65% of the radioactivity was lost in methane probably as a result of fermentation of protozoa that had died. The conclusions are that the contribution of protozoa to microbial protein entering the abomasum is small and a large proportion of the protozoa apparently die and are degraded in the rumen.

MATERIALS AND METHODS

Experimental animals

Six Merino-Border Leicester wethers (approximately 15 months of age and weighing 40–45 kg) were used. The sheep had cannulas in both the rumen and abomasum (approximately 100 mm from the pylorus). They were given (g/d) 700 oaten chaff, 100 lucerne, 100 dried molasses, delivered in equal portions at hourly intervals by a continuous-belt feeding machine.

Experimental procedures

A number of in vitro and in vivo studies were undertaken and these studies were often made simultaneously.

Measurement of the rates of incorporation of [^{14}C]choline into protozoa. Rumen fluid (200 ml) was collected into a plastic syringe by gentle suction from a gauze-covered probe positioned in the dorsal sac of the rumen. This was transferred to a 250 ml conical flask previously filled with CO_2 and fitted with a stopper and one-way gas valve and containing 5 μCi, 0·2 mg [$^{14}CH_3$]choline. The flask was again gassed with CO_2 and then incubated at 39° in a water-bath and shaken at eighty times/min. Fluid samples (10 ml) were taken at intervals whilst gassing with CO_2 for isolation of protozoa and assay of their radioactivity and N content.

Preparation of ^{14}C-labelled protozoa. The preparation of labelled protozoa eventually used was similar to that described by Leng *et al.* (1981). Rumen fluid (100 ml) was incubated with [^{14}C]choline (1 mg, 50 μCi) under CO_2 for 1 h at 39° and shaken at eighty strokes/min. The fluid was then transferred to two 50 ml centrifuge tubes (warmed to 39°) and centrifuged at 500 ***g*** for 1 min. The supernatant fraction was quickly removed by suction and rumen fluid (collected a few minutes previously from the same animal) was added and the protozoa were resuspended by gentle mixing using a vortex mixer; the washing procedure was repeated and the protozoa in approximately 100 ml rumen fluid were then transferred to

a 125 ml stoppered flask that had previously been gassed with CO_2. This labelled protozoal suspension was then ready for injection into the rumen of sheep, or for use in in vitro studies.

In vitro studies with ^{14}C-labelled protozoa. To study the viability of labelled protozoa in vitro studies were undertaken. Immediately following preparation, ^{14}C-labelled protozoa were mixed with 200 ml freshly-collected rumen fluid and incubated for 6 h under CO_2 in a shaking water-bath at 39°. Samples (10 ml) were removed at intervals from the flasks (whilst gassing with CO_2 and continuously mixing) for determination of radioactivity and N in protozoa.

In vivo studies of the dynamics of protozoa in the rumen. Washed ^{14}C-labelled protozoa, prepared from 100 ml rumen fluid, were resuspended in rumen fluid (usually approximately 100 ml) which was injected into the animal from which the protozoa had previously been obtained. Approximately 15 min had elapsed between the end of the incubation period and injection of the labelled protozoa into the rumen.

Rumen fluid, rumen gas and abomasal digesta samples were collected at intervals for up to 3 d.

The quantity of radioactivity as ^{14}C-labelled protozoa injected was determined as follows: 1 ml samples (four replicates) were removed while the injection solution was continuously mixed and placed in 15 ml centrifuge tubes, rumen fluid (9 ml collected a few minutes previously) was added to each tube and the sample mixed and centrifuged at 1000 ***g*** for 1 min, the supernatant fraction was removed and the protozoa and plant debris were resuspended in 10 ml formal saline (40 g formaldehyde/l saline (9 g sodium chloride/l)). This procedure was repeated twice with isotonic saline as the suspending medium. Care was taken not to lose any of the pellet of protozoa and plant debris. After the final washing the pellet of protozoa and plant debris was transferred to a 25 ml volumetric flask and made to volume, 0·5 ml of this suspension was assayed for radioactivity as described by Leng *et al.* (1981).

Estimation of rumen fluid volume, outflow rate and the rate of flow of fluid and particulate matter to the abomasum. In all experiments a continuous infusion of CrEDTA was made into the rumen for 2 d beginning 1 d before the injection of labelled protozoa in order to monitor fluid dynamics. At the end of the study ^{51}CrEDTA (Downes & McDonald, 1964) was injected to estimate volume and the half-time ($t_{\frac{1}{2}}$) of rumen fluid.

In four sheep, 1 week after the kinetics of protozoa had been studied, the flow of abomasal fluid and particulate matter was measured using continuous infusions of CrEDTA and ruthenium phenanthroline (Ru-P) for 3 d (see Tan *et al.* 1971) using the approach of Faichney (1975) as described by Kempton *et al.* (1979).

In vivo and in vitro studies of the metabolism of ^{14}C-labelled dead protozoa. To examine the metabolism of protozoal cells, ^{14}C-labelled protozoa that had been allowed to die were injected into the rumen of two sheep. Protozoa were labelled as described, washed three times with saline, resuspended in 50 ml saline and then placed in a refrigerator overnight (12 h). As far as could be ascertained the protozoa were dead when warmed and examined under the microscope. A sample was taken for estimation of radioactivity, a small amount was introduced into 200 ml of rumen fluid for the in vitro study (see above), and the remainder was injected into the rumen of a sheep on the experimental diet. These in vivo and in vitro studies were carried out in the same way as the studies with live labelled protozoa.

Metabolism of [^{14}C]choline in the rumen. [$^{14}CH_3$]choline (500 nCi) was injected into the rumen of a sheep on the experimental diet. Gas samples for estimation of the specific radioactivity of methane were taken at short intervals of time after injection. Rumen fluid and abomasal digesta samples were taken at approximately 2 h intervals for up to 24 h.

Estimation of methane production in the rumen. Methane production rates were measured

at the same time as digesta flow studies were being made. $^{14}CH_4$ was infused into the sheep for 12 h according to the methods of Murray *et al.* (1978). Samples were obtained over the infusion period from rumen gas for the determination of specific radioactivity of methane.

ANALYTICAL METHODS

Sampling procedures

Rumen fluid. Samples of rumen fluid were taken by suction through a gauze-covered cage positioned in the dorsal rumen by a stainless-steel tube which passed through the bung of the cannula. The samples were placed in two 10 ml centrifuge tubes and immediately processed to isolate protozoa.

For assay of specific radioactivity of rumen CO_2, 5 ml rumen fluid were placed in a McCartney bottle and the CO_2 isolated as barium carbonate as described by Leng & Leonard (1965).

Abomasal digesta were obtained from a single 'T'-piece cannula in the abomasum. All samples were stored at −15° until assayed.

Rumen gas samples. Gas samples were taken by suction into a 100 ml glass syringe from a gauzed-covered tube on the end of a stainless-steel tube inserted in the rubber bung of the cannula. The tube was bent such that it entered the gas phase in the rumen. The gas was transferred to a 25 ml plastic, disposable syringe through a liquid trap and a 16 gauge needle and appropriate plastic tubing. The 25 ml syringe was connected by plastic tubing to a 5 ml syringe containing 3 ml CO_2-free sodium hydroxide (50 g/l) which was transferred to the larger syringe by pressure. The syringe was shaken to absorb CO_2 and left for up to 12 h. Although some of the methane was lost from the plastic syringes when samples were left for several days there was still sufficient methane for analysis.

Isolation of and assay of radioactivity in protoza and radioactivity in protozoa-free rumen fluid

Rumen fluid (10 ml) was centrifuged at 500 ***g*** for 1 min. Of the supernatant fraction 0·5 ml was then placed in a scintillation vial with 10 ml of a scintillation cocktail and assayed for radioactivity (see Leng *et al.* 1981). The supernatant fluid was then removed by suction and the pellet resuspended in 10 ml formal saline, this was then centrifuged at 500 ***g*** for 1 min and the supernatant fraction removed. This washing procedure was repeated but using saline. The upper 'particles' layer on the sedimented protozoa was removed during each washing and the protozoa were separated from the heavy food materials, which settled quickly in the mixture, by decanting the suspended protozoa into a second tube before centrifugation. After three washings the protozoa were essentially free from feed particles when examined under the microscope. Following the third washing the protozoa were suspended in approximately 2 ml saline and whilst mixing on a vortex mixer a 0·5 ml sample was taken for assay of radioactivity and a second 0·5 ml for the determination of N. A further 0·5 ml of sample was added to 5 ml formal saline for enumeration of protozoa.

Assay of radioactivity in abomasal digesta

Abomasal digesta were assayed for radioactivity in two ways. Mixed digesta (1 g) was added to 10 ml of the scintillation mixture used by Leng *et al.* (1981) and following an overnight period for extraction of radioactivity into the scintillant the vial was counted. The second method was as follows. Approximately 3 ml mixed digesta were added to a tarred scintillation vial and weighed and dried at 95°. Then 5 ml commercial solubilizer, NCS™ (supplied by The Radiochemical Centre, Amersham, UK) were added and the sample left overnight. The sample was centrifuged and 4 ml counted in 10 ml scintillation cocktail.

For estimation of radioactivity in abomasal particulate materials and liquid the following

procedures were employed: abomasal digesta (10 ml) were centrifuged at 20000 *g* for 20 min and 0·5 ml supernatant fraction placed in a scintillation vial with 10 ml scintillation mixture (Leng *et al.* 1981) and counted. The supernatant fraction was removed, the tubes inverted on filter paper to drain, and the pellet was then mixed with saline and made up to 10 ml; 0·5 ml of this was taken for assay of radioactivity as described for digesta.

Recovery of ^{14}C-labelled protozoa from abomasal digesta was assayed as follows: ^{14}C-labelled protozoa (approximately 60 nCi, 1 mg N) prepared as indicated previously was added to 10 ml abomasal digesta and incubated for 15 min at 39°; 1 ml digesta was taken and added to 10 ml scintillant and assayed for radioactivity, then the sample was partitioned into fluid and particulate matter as described previously. A ^{14}C-labelled protozoa sample was added to a further 10 ml sample abomasal digesta and the digesta, fluid and particulate matter assayed for radioactivity after solubilizing in NCS™ as before. In both instances the recovery of radioactivity was quantitative.

Estimation of specific radioactivities of methane and CO_2 in rumen gas

Samples of rumen gas were stored in 25 ml syringes containing 3 ml NaOH for 12 h. The methane was oxidized to CO_2 in a tube furnace containing ferric oxide and maintained at 720°. The gas sample was injected directly into the furnace and was washed through the furnace using a continuous stream of N_2 gas (3 ml/min). Effluent gases were mixed with 0·5 M-NaOH (CO_2-free; 10–15 ml) infused at a 'T'-piece so as to form alternate gas and alkali bubbles which were passed through a glass tube in the form of a spiral (see Loughnan, Davis & Leng, unpublished results). The effluent was collected in a test-tube and the absorbed CO_2 was precipitated as $BaCO_3$ by adding 2 ml ammonium hydroxide (50 g/l) followed by 3 ml barium chloride dihydrate (200 g/l). The precipitate was washed, dried, weighed and assayed for radioactivity as described previously (Leng & Leonard, 1965).

CHEMICAL METHODS

Scintillation counting of radioactive digesta samples

All samples were counted using a Packard Tricarb Scintillation Spectrometer in a toluene–Triton X100 (2:1, v/v) cocktail containing PPO (1 g/l) and POPOP (0·4 g/l). Efficiencies were determined by 'spiking' samples with 0·1 ml of a standard [^{14}C]choline solution and also using quenched correction with external standardization. Backgrounds for each type of preparation were obtained by counting 'cold' samples prepared before injection of labelled materials.

Routine analysis

Dry matter of abomasal digesta was estimated following drying at 80° for 48 h. The method for estimation of N content in protozoa has been described previously (Leng *et al.* 1981). Cr in rumen fluid and abomasal digesta was estimated using an atomic absorption spectrometer (Model 360G; Perkin Elmer, Norwalk, Connecticut, USA). ^{103}Ru in abomasal digesta was assayed as described by Tan *et al.* (1971). VFA concentration and proportions in rumen fluid were estimated using gas–liquid chromatography (Erwin *et al.* 1961) and isocaproic acid as an internal standard. Ammonia in rumen fluid was estimated according to Nolan & Leng (1972) and differential protozoal counts were done according to Warner (1962).

Calculations

Calculation of volume, $t_{\frac{1}{2}}$ and flow-rate of rumen fluid were made from the concentrations of Cr or ^{51}Cr in rumen fluid following infusions or injections of the respective marker assuming first-order kinetic processes applied to both results from infusion (at plateau concentrations) and single injections (see Downes & McDonald, 1964).

The pool size, $t_{\frac{1}{2}}$ and apparent production rates of protozoa were calculated according to the methods of Leng *et al.* (1981).

Methane production rate was calculated from the specific radioactivity of methane when this had reached a plateau level during infusion of $^{14}CH_4$ (see Murray *et al.* 1978).

Calculation of the routes of excretion of ^{14}C following injection of ^{14}C-labelled protozoa into the rumen

In these studies ^{14}C-labelled protozoa were injected into the rumen and the specific radioactivity of methane, abomasal fluid and abomasal particulate digesta estimated over a period of time (see Fig. 5). Following a period for mixing and clearance of contaminating ^{14}C (i.e. ^{14}C in the mixture which was not bound in protozoa and which was less than 5% of the ^{14}C in protozoa), the radioactivity in the various pools apparently declined according to first-order processes. The 'build-up' curves for specific radioactivities in abomasal fluid and abomasal particulate digesta were not readily apparent (mainly because insufficient samples could be taken over the early part of the study). The 'build-up' curve in methane was extremely rapid and again could not be easily defined.

The equations for the relationships of specific radioactivities with time of samples taken from the various pools between 2 h and up to 3 d were of the general form:

$$SR_t = SR_0 e^{-mt},$$

where SR_t is specific radioactivity at time t, SR_0 is the zero time intercept and m is the rate-constant.

The area (A) under a curve described by a single exponential function was estimated by integrating the equations from zero time to infinity i.e.

$$A = \frac{SR_0}{m}.$$

The area under the radioactivity with time-curve of any pool multiplied by the rate of total loss from the pool represents the total quantity of radioactivity passing via that pool. Thus the quantity of radioactivity lost via eructation in methane or via movement in digesta fluid or solids to the lower tract could be calculated.

Radioactive compounds. [$^{14}CH_3$]choline, $^{14}CH_4$, ^{51}CrEDTA and ^{103}Ru P were obtained from the Radiochemical Centre, Amersham, UK.

RESULTS

Measurement of rumen function

The numbers of protozoa and concentrations of ammonia and VFA in rumen fluid are given in Table 1.

The pool size, $t_{\frac{1}{2}}$ and flow-rate of fluid out of the rumen and through the abomasum are shown in Table 2, together with the dry matter flow to the abomasum and the apparent digestibility of dry matter across the rumen. Methane production rates are also given in Table 2.

Uptake and degradation of [^{14}C]choline by protozoa

The pattern of uptake of [^{14}C]choline by protozoa in rumen fluid in vitro is given in Fig. 1. Depending on the concentration of protozoa the uptake of [^{14}C]choline was between 2 and 10%; this was reached after approximately 3 h. Radioactivity in solution also decreased to reach a minimum at approximately the same time. Methane collected from the gas space of the incubation flask was highly radioactive, whereas CO_2 was unlabelled.

Table 1. *Protozoa numbers and rumen fluid metabolites in the experimental sheep*

Expt no.	Rumen protozoa* ($\times 10^{-5}$/ml) Small ciliates‡	Large ciliates§	Ammonia concentration† (mg nitrogen/l)	Total concentration of volatile fatty acids (mM/l)	Volatile fatty acids (mmol/mol) Ac	Prop	But	Others
1	9·5	0·038	115	49	720	180	90	10
2	8·8	0·363	118	38	730	170	80	20
3	35·6	0·350	130	48	740	160	80	20
4	31·0	0·01	132	58	780	140	60	20
5	24·6	0·01	94	53	740	160	80	20
6	10·9	0·01	86	53	720	190	70	20

Ac, acetate; Prop, propionate; But, butyrate; Others, isobutyrate + isovalerate + valerate.
* SE of protozoal counts (on nineteen to thirty samples) was approximately ± 10%.
† SE of ammonia concentrations varied between 2–11% (for approximately twenty to thirty samples).
‡ *Entodinium* spp.
§ *Polyplastron* spp. in Expt 1 and 2 but *Epidinium* spp. in Expt 3.

Table 2. *Methane production and the kinetics of rumen fluid and abomasal digesta*
(Intake of dry matter (DM) was approximately 558 mg/min)

Expt no.	Rumen fluid volume (l)	$t_{\frac{1}{2}}$ rumen fluid (min)	Rumen fluid outflow rate (ml/min)	Abomasal fluid flow (ml/min)	Particulate DM flow in abomasal digesta (mg/min)	Methane production rate (mg carbon/min)
1	4·8	505	6·6	7·6	252	7·6
2	5·1	486	7·3	9·4	294	7·2
3	6·5	564	8·0	7·4	294	6·9
4	3·8	310	8·7	9·0	223	7·2
5	4·7	544	6·7	—	—	—
6	5·0	540	6·5	—	—	—

$t_{\frac{1}{2}}$, half-life.

Effects of washing on protozoal radioactivity

For the isolation of protozoa for determination of radioactivity three washings were required. Since protozoa that were allowed to die were found to 'leak' ^{14}C, it was necessary to check that significant amounts of radioactivity were not lost in the washing procedures. Live ^{14}C-labelled protozoa were added to microbe-free rumen fluid (prepared by centrifugation) and samples taken through the washing procedures. The protozoa were either killed in the first washing by using formal saline, with saline being used in subsequent washings or kept alive by using only saline during all three washings. The radioactivity in the washings and in protozoa is shown in Table 3. No significant loss of ^{14}C occurred with the washing procedures used, provided the washings were carried out without delay. The subsequent leakage of radioactivity with time was substantial (Table 3).

An initial wash with formal saline as compared to saline was beneficial in that it slowed the subsequent loss of radioactivity from the protozoa.

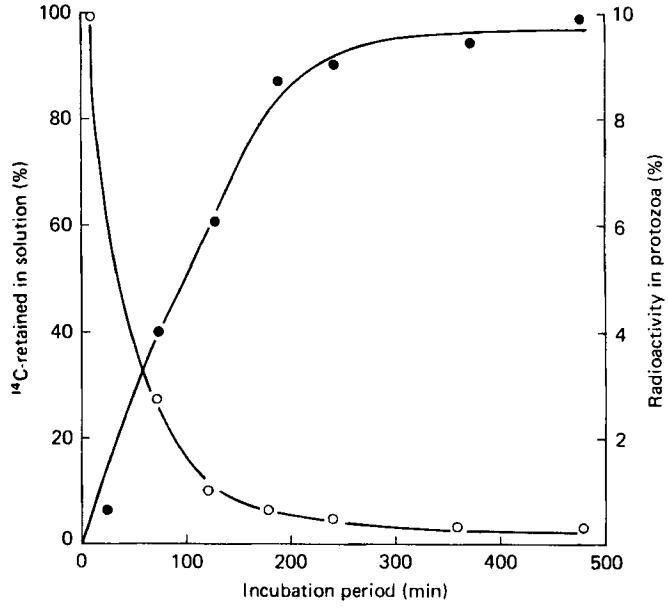

Fig. 1. Percentage of radioactivity in solution (○) and incorporated into protozoa (●) during an incubation of [^{14}C]choline (0·2 mg, 5 μCi) in 200 ml rumen fluid in which there were approximately $1{\cdot}2 \times 10^6$ protozoa present mainly *Entodinium* spp.

Table 3. *The effect of washing protozoa and allowing the preparation to stand in saline on the radioactivity retained in protozoa**

	Radioactivity in solution (μCi/50 ml)		Radioactivity in protozoa (μCi/50 ml)	
	A	*B*	*A*	*B*
Beginning of incubation	3·99	3·99	0	0
End of incubation	0·72	0·72	0·33	0·33
Reconstituted in rumen fluid	0·03	0·03	0·32	0·32
Wash				
1st	0·01	0·01	0·32	0·32
2nd	0·01	0·02	0·32	0·32
Period in saline (min)				
100	0·03	0·11	0·28	0·23
200	0·04	0·13	0·26	0·20
800	0·06	0·16	0·24	0·18

* The study was made with 100 ml sample of rumen fluid which was incubated for 1 h with approximately 8 μCi [^{14}C]choline. At the end of the incubation period the protozoa were washed with rumen fluid and resuspended in 100 ml rumen fluid. The protozoa in half (sample *A*) of this was, on the first occasion, washed with formal saline (40 g formaldehyde/l saline (9 g sodium chloride/l)) followed by saline; the second sample (*B*) was washed only with saline on both occasions. The samples were allowed to stand and samples were taken at intervals for assay of radioactivity in solution and retained in the protozoa.

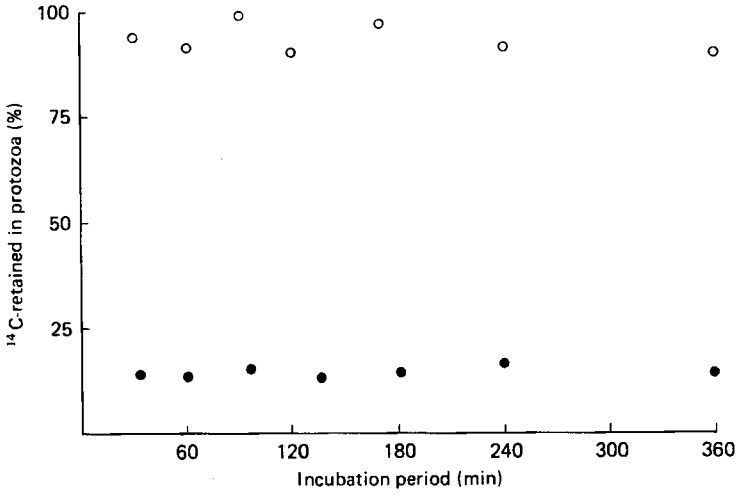

Fig. 2. The percentage retention of ^{14}C in protozoa after inoculation of 200 ml rumen fluid with washed ^{14}C-labelled protozoa prepared as described on p. 400 (○) or washed protozoa that have been killed by storage at 4° for 12 h (●).

Retention of radioactivity in protozoa incubated in rumen fluid in vitro

A small sample (5 ml) of ^{14}C-labelled live protozoa prepared as previously described was added to 200 ml rumen fluid taken from the animal a few minutes previously. The percentage of the initial dose retained in protozoa is shown in Fig. 2. More than 90% of the radioactivity was retained in protozoa after a 6 h incubation. The specific radioactivity of the protozoal pool (μCi/g N) was, however, unchanged over the same time period.

The metabolism of dead protozoa in vitro

^{14}C-labelled protozoa that had died during 12 h storage in saline at 4° were added to 200 ml rumen fluid drawn from the animal a few minutes previously. Approximately 50% of the ^{14}C was present in solution. The percentage uptake of the added radioactivity by protozoa is shown in Fig. 2. Only 10–15% of the radioactivity added as the ^{14}C-labelled protozoa suspension (dead) was retained in the protozoal pool.

The fate of dead protozoa injected intraruminally

The incorporation of radioactivity into the protozoa and methane when a preparation containing dead ^{14}C-labelled protozoal preparation (50% ^{14}C in solution) was injected into the rumen is shown in Fig. 3.

The radioactivity in methane increased rapidly to a peak at 40 min and then decreased to negligible quantities by 200 min. Negligible amounts of radioactivity were present in abomasal digesta but radioactivity appeared in the protozoal pool and could be detected for up to 15 h (Fig. 3).

Metabolism of [^{14}C]choline in the rumen

As a small amount of [^{14}C]choline was injected into the rumen with all labelled protozoal preparations, it was essential to estimate the extent of breakdown of the choline and also whether significant labelling occurred in protozoa from this source. For this reason 500 nCi

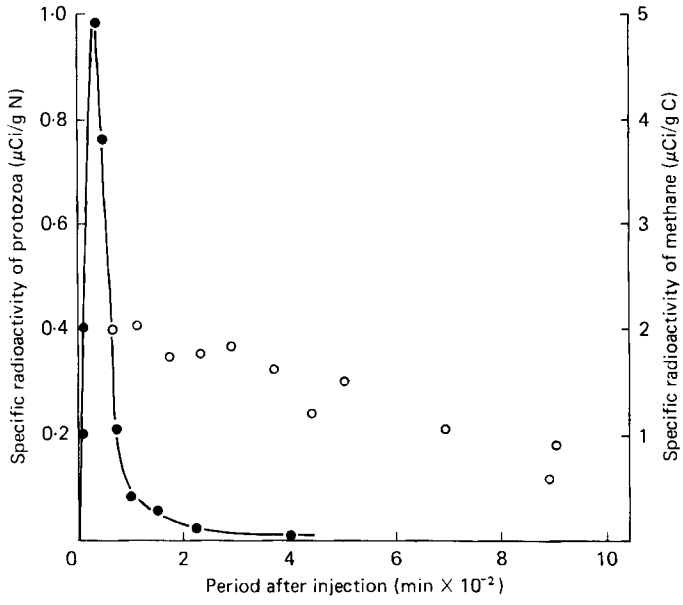

Fig. 3. The specific radioactivity of protozoa (μCi/g nitrogen) (○), and methane (●) (μCi/g carbon) following injection of 4·7 μCi ^{14}C-labelled, dead protozoa into the rumen of a sheep.

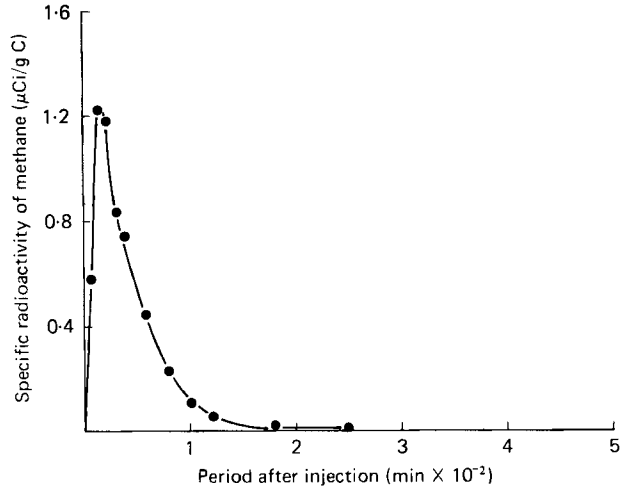

Fig. 4. The specific radioactivity of methane (μCi/g carbon) following an injection of [^{14}C]choline (500 nCi, 1 mg) into the rumen of a sheep.

(at least three to six times the amount present in labelled protozoa preparations) of [^{14}C]choline in 25 ml water were injected into the rumen and the ^{14}C in methane and protozoa estimated. Protozoa were not detectably labelled and no radioactivity was found in abomasal digesta. The radioactivity in methane (Fig. 4) followed the same pattern as that for injection of dead ^{14}C-labelled protozoa (Fig. 3).

Table 4. *Dynamics of protozoa in the rumen of sheep*

Expt no.	Injected dose (μCi)	Protozoal pool size (g nitrogen)	Apparent production rate protozoa (mg N/min)	$t_{\frac{1}{2}}$ protozoa (min)
1	1·68	1·92	1·72	772
2	1·26	1·74	1·48	813
3	2·53	5·72	4·11	966
4	1·71	2·64	2·63	696
5	1·67	1·70	1·25	941
6	2·69	1·31	1·03	885

$t_{\frac{1}{2}}$, half-life.

Apparent irreversible loss rate (production rate) and the pool size and $t_{\frac{1}{2}}$ *of protozoa estimated using* ^{14}*C-labelled protozoa*

Following injection of the ^{14}C-labelled protozoa the specific radioactivity of protozoa (μCi/g N) declined according to first-order kinetic processes (see Fig. 5) which was followed in some instances for up to 3 d.

The $t_{\frac{1}{2}}$ of ^{14}C-labelled live protozoa and their apparent irreversible loss rate or production rate and pool size were calculated as for first-order kinetic processes. The results for six sheep are given in Table 4.

Irreversible loss of radioactivity from the protozoal pool in the rumen

Considerable ^{14}C was lost in methane and via abomasal digesta (fluid and particulate matter). The specific radioactivity of methane (μCi/g C) and abomasal fluid (μCi/100 ml) and particulate materials (μCi/0·1 g dry matter) declined at similar rates to those of the protozoa (Fig. 5, Table 5). The apparent irreversible losses of ^{14}C (1) in methane and (2) by movement out of the rumen in both liquid and particulate material are given in Table 5 and the calculated percentage loss of ^{14}C via these three routes in Table 6.

DISCUSSION

In earlier studies on the turnover of protozoa in the rumen (Leng *et al.* 1981) large ciliate protozoa were studied and their size facilitated separation from rumen fluid. In the studies reported here, isotope methods are now described for studying the growth and loss of small ciliate protozoa (largely *Entodinia*) in the rumen of sheep and of the movement of ^{14}C to the abomasum. The techniques developed are now discussed.

The ^{14}*C-labelled protozoa preparation*

Initial studies were made of the rate of incorporation of [^{14}C]choline into protozoa; incorporation was rapid but, in contrast to results obtained with large ciliates in cattle on sugar-cane-based diets (see Leng *et al.* 1981), [^{14}C]choline was also rapidly lost from the rumen fluid. Methane collected from the incubation medium was found to be radioactive indicating that this was the major end-product of choline fermentation which had been shown previously by Neill *et al.* (1978) and Neill *et al.* (1979). Initial studies indicated that approximately 10% of [^{14}C]choline added to rumen fluid was incorporated during an incubation period of 2–4 h, but it was felt that the shortest possible period of incubation would be most likely to ensure viable labelled protozoa. An incubation period of

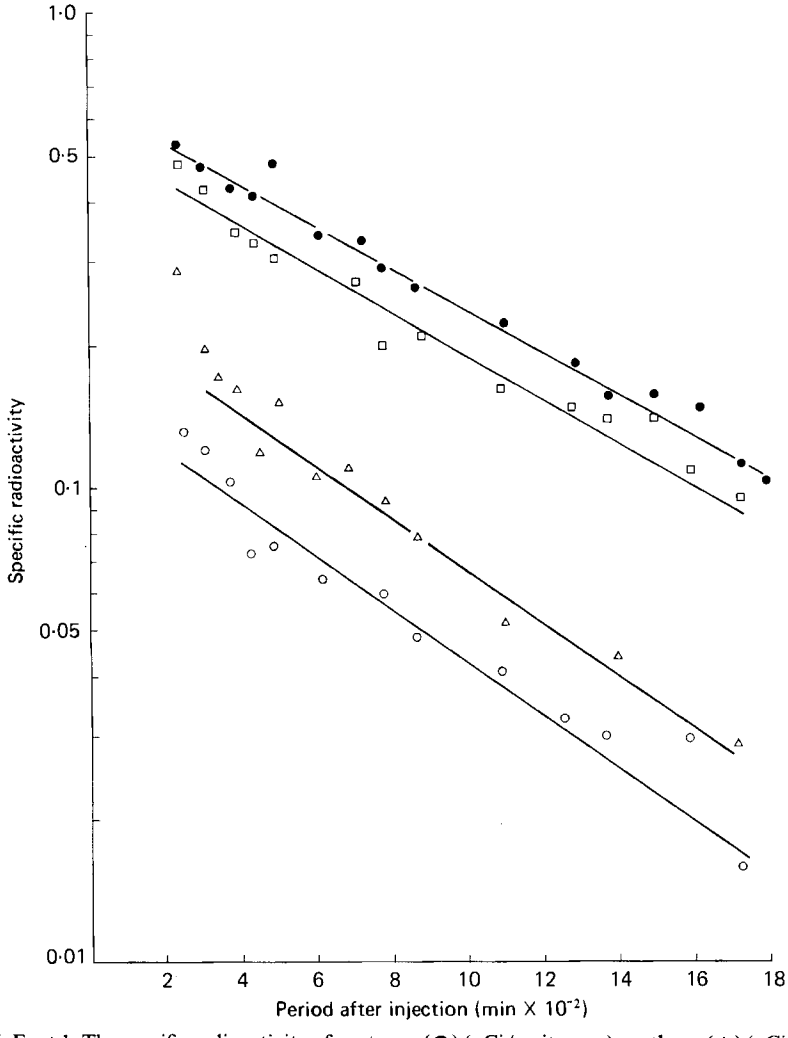

Fig. 5. Expt 1. The specific radioactivity of protozoa (●) (μCi/g nitrogen), methane (△) (μCi/g carbon), abomasal fluid (□) (μCi/100 ml) and abomasal particulate matter (○) (μCi/0·1 g dry matter) following a single injection of ^{14}C-labelled protozoa (1·71 μCi) prepared from 100 ml rumen fluid incubated with 50 μCi [^{14}C]choline.

approximately 1 h allowed 1–4 μCi (2–8% of added ^{14}C) of ^{14}C-labelled protozoa to be collected from 100 ml rumen fluid after incubation and this was sufficient for the studies.

Contamination of the ^{14}C-labelled protozoa preparation

Protozoa labelled in vitro remained viable after three washings with freshly-collected rumen fluid, however two washings were sufficient for present purposes and were used throughout the present study in order to safeguard their viability. The rapid fermentation of the methyl groups of choline to methane (see Neill *et al.* 1978) ensured a low level of radioactivity in the medium at the end of the incubation period. Two washings with rumen fluid reduced this contamination to 0·05–0·1 μCi/100 ml which was less than 5% of the radioactivity present in protozoa (Table 3). In order to determine whether this unavoidable contamination

Table 5. *The specific radioactivity at zero time (SR_0) and rate constants* (m; */min* × 10^3) *of the isotope-dilution curves of protozoa, methane, abomasal fluid and abomasal particulate matter following a single injection of* ^{14}*C-labelled protozoa*

(The calculated areas under the isotope-dilution curves A, (SR_0/m) and the radioactivity irreversibly lost from each pool, *IR* (μCi) are also given)

		Protozoa				Methane			
Expt no.	Injected dose (μCi)	SR_0 (μCi/g nitrogen)	*m*	*A*	*IR*	SR_0 (μCi/g C)	*m*	*A*	*IR*
1	1·68	0·88	0·90	975	1·68	0·22	1·22	185	1·41
2	1·26	0·72	0·85	850	1·26	0·15	1·24	120	0·86
3	2·53	0·44	0·71	619	2·53	0·28	1·22	230	1·58
4	1·71	0·65	0·99	655	1·71	0·24	1·25	196	1·41
		Abomasal digesta fluid				Abomasal digesta particulate matter			
Expt no.	Injected dose (μCi)	SR_0 (μCi/l)	*m*	*A*	*IR*	SR_0 (nCi/g DM)	*m*	*A*	*IR*
1	1·68	0·051	1·29	39	0·30	2·64	2·40	1100	0·28
2	1·26	0·046	0·90	51	0·48	1·14	0·90	1267	0·37
3	2·53	0·056	1·16	48	0·36	1·94	1·13	1713	0·50
4	1·71	0·055	1·10	51	0·46	1·53	1·25	1228	0·27

DM, dry matter.

Table 6. *Radioactivity irreversibly lost from protozoa via methane or passage down the digestive tract in fluid or in particulate dry matter*

Expt no....	1	2	3	4
^{14}C injected (μCi)	1·68	1·26	2·53	1·72
^{14}C in methane				
μCi	1·41	0·86	1·58	1·41
% total	71	50	65	66
^{14}C in abomasal fluid				
μCi	0·30	0·48	0·36	0·46
% total	15	28	15	21
^{14}C in abomasal digesta particulate matter				
μCi	0·28	0·37	0·50	0·27
% total	14	22	20	13
Total				
μCi	1·99	1·71	2·44	2·14
% total injected*	118	136	96	124

* Calculated recovery of radioactivity in methane, plus abomasal fluid and particulate matter as a percentage of dose injected.

had any influence on the specific radioactivities of the various pools monitored, 0·5 μCi [^{14}C]choline was injected directly into the rumen. This choline was rapidly degraded to methane, supporting the findings of Neill *et al.* (1979). No detectable radioactivity appeared in isolated protozoa or abomasal digesta. No significant radioactivity was present in either rumen fluid or methane 2–3 h after injection (see Fig. 4). The contamination of ^{14}C in

solution in the injected dose of labelled protozoa apparently had no influence on the specific radioactivities of protozoa or abomasal digesta and its presence could be ignored. However the values for methane specific radioactivity in samples taken up to 2 h after commencement of the studies were affected and values obtained up to 3 h post injection were not included in any mathematical analysis.

Viability of labelled protozoa

^{14}C-labelled protozoa prepared as discussed previously and washed three times with saline were killed by holding them for 12 h at 4°. When these were incubated with rumen fluid or injected into the rumen their radioactivity was rapidly lost via methane (see Fig. 3). The radioactivity retained in the live protozoa when dead ^{14}C-labelled protozoa were incubated in vitro was approximately 10% of the radioactivity added (Fig. 2), suggesting that the live protozoa present salvaged choline from dead protozoa but did not exclude retention of ^{14}C in dead protozoa debris. This was supported by in vivo studies in which a small proportion of radioactivity from the dead protozoa was apparently re-incorporated into the resident population (Fig. 3). However, if some of the protozoal materials are not readily fermented in the rumen then a proportion of the ^{14}C in isolated protozoa could still have arisen from this source. The $t_{\frac{1}{2}}$ of the protozoa that had become labelled by injection of the dead ^{14}C-labelled protozoa in this animal was similar to that obtained after injection of live labelled protozoa (Fig. 3, Table 4). The amount of radioactivity in the protozoal pool following injection of the dead protozoal preparation was calculated as follows. On the basis of the protozoal numbers found in rumen samples, the protozoal pool in the rumen may have been approximately 1·5 g N (see Table 4) and therefore 0·6 μCi ^{14}C-labelled protozoa were present in the protozoal pool in the rumen from a total dose of injected dead ^{14}C-labelled protozoa of 4·7 μCi. A large proportion of the radioactivity was apparently lost via methane (Fig. 3) with little passing down the digestive tract to the abomasum.

Freshly-prepared ^{14}C-labelled protozoa were still viable when incubated for 6 h in rumen fluid at 39° since there was only a small decrease in total radioactivity in the protozoal pool (Fig. 2). The protozoa in these incubations apparently did not grow since the specific radioactivity (μCi/g N) of protozoa was constant over the 6 h period also indicating that there was no apparent loss of the [^{14}C]choline from those protozoa. The small loss of radioactivity from the protozoal pool is consistent with the whole animal studies and suggests some distintegration of protozoa, probably after death of a proportion of the protozoa. The apparent lack of growth of protozoa has been observed (Ffoulkes & Leng, unpublished results) with rumen fluid from cattle and may be due to a rapid exhaustion of an essential nutrient in vitro which would normally be provided in the diet or conceivably by the animal. Since choline is rapidly fermented in rumen fluid, this is a possible nutrient deficiency in vitro. However, the in vitro studies were usually conducted in the mid-morning period and Warner (1962) showed that, in sheep fed once daily, protozoal division was synchronized to occur before the morning feed. This suggests a sporadic growth of protozoa, but this is not borne out by the present in vivo studies in which there was no indication of periodic increases in the rate of dilution of the ^{14}C-labelled protozoa (see Fig. 5).

In subsequent studies protozoa isolated from rumen fluid following a 1 h incubation period and then resuspended in protozoa-free rumen fluid incorporated the same amount of radioactivity from [^{14}C]choline as protozoa incubated without isolation and preincubation (Leng, unpublished results). *In toto* these studies indicate that the ^{14}C-labelled protozoa were viable and, so far as could be ascertained, behaved normally in the rumen.

Rumen volume, pool size and $t_{\frac{1}{2}}$ *of fluid*

Simultaneously with studies of protozoal turnover, a continuous infusion of CrEDTA was made and the concentration of Cr in rumen and abomasal fluid was used to calculate fluid flow from the rumen and through the abomasum respectively. Other workers have used the decline in concentration of Cr in rumen fluid following cessation of the infusion, to calculate the $t_{\frac{1}{2}}$ of fluid and its pool size. In the studies presented here it was found convenient to measure these factors using a single injection of ^{51}CrEDTA (Downes & McDonald, 1964) but the two markers (i.e. ^{51}Cr and Cr) gave comparable results.

Pool size, $t_{\frac{1}{2}}$ *and apparent irreversible loss (production rate) of protozoa*

The number of protozoa in rumen fluid samples taken over 2 or 3 d of each experiment varied considerably and this is consistent with some protozoa being attached to digesta particles or sequestered in the rumen. The diet used was designed to promote large rumen populations of protozoa through the inclusion of molasses in the diet and protozoal populations were consistently high. This facilitated their isolation in a relatively pure form. The main species present were small *Entodinia* but in one animal significant numbers of *Epidinium* spp were present and in two others *Polyplastron* spp were present. Because of their size (approximately 100 times that of *Entodinia* spp) these large ciliate protozoa were a large proportion of the protozoal biomass when they were present in excess of 10^4/ml. The largest pool of protozoa in these sheep was associated with large populations of *Entodinia* and *Polyplastron* spp (sheep no. 3, Tables 1 and 4). The $t_{\frac{1}{2}}$ of ^{14}C-labelled protozoa was consistently greater than the $t_{\frac{1}{2}}$ of rumen fluid (Tables 2 and 4) which indicates a preferential retention of protozoa in the rumen and supports the conclusions of Weller & Pilgrim (1974) and Harrison *et al.* (1979). However, from the dilution of labelled protozoa, between 2 and 4 g N as protozoa were produced daily which is likely to represent approximately 12–24 g protein. The calculated microbial protein leaving the rumen in sheep on this diet (consuming 400 g digestible organic matter daily) is 12 g N or 75 g protein (assuming 30 g N as microbial protein are available per kg apparently-fermented organic matter (Roy *et al.* 1977)). Protozoa may thus synthesize up to one-third of the net microbial protein produced in the rumen of these sheep.

In these studies a single exponential function best described the relationship between the specific radioactivity of protozoa (μCi/g N) and time. The longest experiment lasted 3·5 d and a second exponent could not be detected in the specific radioactivity-time relationship, suggesting that the choline in structural components of protozoa, which was apparently degraded in the rumen, was not recycled by incorporation into other live protozoa. This is contrary to the finding of incorporation of ^{14}C into protozoa when dead, labelled protozoa were injected into the rumen. This is difficult to reconcile but it is possible that protozoa that die and are degraded are at different sites from the injected protozoa.

Routes of loss of protozoa

A production rate of protozoa of 1·4–5·8 g N/d indicates a loss rate of a similar magnitude. Some indication of the routes of loss of protozoal cells can be obtained if the fate of the ^{14}C in protozoa can be followed.

In vitro studies indicated that the turnover and loss of choline from protozoa was undetectable during a 6 h incubation period (specific radioactivity remained constant). This supports the concept that the choline in live protozoa is not appreciably lost to the medium (see also Neill *et al.* 1978) and that the decline in specific radioactivity of protozoa in the rumen which has been labelled by injection of ^{14}C-labelled live protozoa represented the rate of growth of protozoa. ^{14}C in the labelled protozoa is present largely as [^{14}C]phos-

phatidylcholine (see Broad & Dawson, 1976). Studies with dead protozoa suggested that the methyl groups of choline in the components of protozoa are readily converted to $^{14}CH_4$ in the rumen. Thus there appear to be two major routes of loss of ^{14}C from protoza: (1) by transfer to the post-ruminal tract in digesta (as protozoa or protozoal debris) or (2) release of ^{14}C following death and degradation of protozoa in the rumen with conversion of the labelled products to methane. Following injection of labelled protozoa into the rumen ^{14}C appeared in both methane and abomasal digesta. The radioactivity in rumen fluid was close to that in abomasal fluid indicating that some of the radioactivity in abomasal fluid arose from rumen fluid. The high specific radioactivity of methane following injection of ^{14}C-labelled protozoa (at a time when any contaminating radioactivity in the injection mixture would have been cleared) suggests that a considerable turnover of protozoa occurred in the rumen.

The approximate loss of radioactivity via methane or passage down the tract in digesta was calculated from the area under each of the secondary isotope dilution curves following injection of ^{14}C-labelled protozoa. There are a number of difficulties in making these calculations which make them approximate i.e. the areas under the isotope-dilution curves should take into consideration the 'build-up' components (see p. 410). The 'build-up' components of the specific radioactivity–time relationships for abomasal digesta or methane could not be determined, and therefore a single exponential function for each relationship over-estimates all routes of loss of radioactivity. This is borne out by the results given in Table 6 which shows that the sum of all these routes of loss of radioactivity over-estimates the total loss by approximately 19%. However, it is quite definite that a large proportion of the ^{14}C lost from the protozoal pool (approximately 65% of the total) was lost as methane suggesting that the majority of the loss of protozoa from the rumen occurs through death and fermentation of protozoa in the rumen. Protozoal death and lysis in the rumen are likely to result in fermentation of much of the protozoal protoplasm and other soluble components to VFA and ammonia. As a result most of the protozoal protein synthesized in the rumen does not become available for digestion to amino acids and absorption from the lower digestive tract.

Bird *et al.* (1978) using sheep with mainly small *Entodinia* in the rumen, and Minor *et al.* (1977) using cattle with mainly large cilitate protozoa, found few protozoa in omasal contents relative to those present in the rumen. Bird *et al.* (1978) also showed that protozoa incubated in omasal contents in vitro were still viable at the end of 3 h incubation. Dawson *et al.* (1981) however, examined the omasum contents taken from killed sheep and found numbers of viable rumen ciliated protozoa which led them to suggest that the unusual ionic conditions apertaining in this organ caused the protozoa to die and their phosphatidylcholine to be degraded by an autolytic phospholipase present (Broad & Dawson, 1976). Although this is possible and to some extent is supported by the apparent lack of recycling of choline in the rumen in these studies, it would be necessary for the methyl groups of the choline to be converted by microbes to methane in the omasum and for this to leak back into the rumen through the recticulo-omasal orifice. Gases produced in the omasum do move back to the reticulum and are eructated (Svendsen, 1975). However, the smaller numbers of protozoa observed by Weller & Pilgrim (1974), Bird *et al.* (1978) and Minor *et al.* (1977) in omasal relative to rumen contents suggest that this explanation could only account for a small proportion of the loss from the protozoal pool. The sequestration of holotrich protozoa on the wall of the reticulum in the cattle after 1 d without food (Abe *et al* 1981) is also strong support for retention of protozoa in the forestomachs.

Conclusions

These studies suggest that when small ciliate protozoa are present in large numbers they may contribute a substantial proportion of the microbial protein synthesized in the rumen,

but that the majority of the protozoal cells are probably degraded in the rumen. In this way they lower the quantity of protein moving to the lower digestive tract. In addition the engulfment of bacteria by protozoa is likely to reduce further microbial amino acid availability. This study suggests that the 16% increase in protein availability on defaunation of sheep observed by Lindsay & Hogan (1972) is likely to be much higher whenever protozoa numbers reach levels similar to those present in the sheep used in the present studies. The suggestion made here of only a small proportion of protozoa present in the rumen moving into the lower digestive tract is well supported by the studies of Dawson *et al.* (1981) who showed that little or no labelling occurs in the blood lipids when sheep are fed on [^{14}C]choline for substantial periods of time. The studies of Dawson and his colleagues (Dawson *et al.* 1981) together with our own, suggest that at least some choline would have been salvaged from the diet by protozoa but this does not appear to reach the lower digestive tract.

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