Kinetics of large ciliate protozoa in the rumen of cattle given sugar cane diets

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1. Experiments were undertaken to examine the kinetics of large ciliate protozoa in the rumen of cattle on sugar-cane diets.

2. Three Zebu bulls were fed once daily on a diet of sugar cane and wheat bran. The diurnal patterns of volatile fatty acids and ammonia concentrations, and the numbers of protozoa in rumen fluid were determined. The numbers of protozoa reached values of 5×10^4/ml for holotrichs (large ciliates) mainly *Isotricha* and *Dasytricha* spp and 4×10^5 for smaller protozoa, mainly *Entodinia* (small ciliates).

3. A method was developed which allowed large ciliate protozoa in rumen fluid to be separated from plant material and bacteria and concentrated in a relatively uncontaminated form. Analysis of these protozoa indicated that 1.8×10^5 large ciliates contained 1 mg nitrogen and approximately 32 mg dry matter.

4. A labelled preparation consisting mainly of large ciliates (principally *Isotricha* spp.) was obtained by incubating isolated protozoa in rumen fluid (free of plant materials) containing [^{14}C-methyl]choline and then isolating them by sedimentation and differential centrifugation.

5. A portion of the preparation containing labelled protozoa was incubated in vitro with rumen fluid to determine the turnover of ^{14}C-labelled metabolites. There was no apparent dilution of the label in the protozoa over a 22 h period.

6. A major portion of the preparation containing labelled protozoa was returned to the rumen of each of the donor cattle as a single injection. The specific radioactivity in the large protozoa (μCi/mg N) was monitored frequently for over 30 h, and thereafter daily for a further 12 d. The kinetics of tracer dilution were analyzed to give estimates of the size of the pool of these large ciliates in the rumen (24–46 g N), and of their apparent rate of turnover.

7. In contrast to the slow turnover of the large ciliates, the rate of turnover of the rumen fluid pool (approximately 54 l), estimated from the rate of dilution of polyethylene glycol, was considerably faster. Large ciliates were therefore selectively retained within the rumen.

Involvement of protozoa in the nutrition of ruminants has been studied mainly in animals in which small ciliate protozoa (*Entodinia* spp.) rather than the large ciliate protozoa (*Holotricha* spp.) were predominant in the population. The smaller protozoa actively engulf bacteria (Coleman, 1975), and do not pass out of the rumen in proportion to their concentration in rumen fluid (Weller & Pilgrim, 1974; Harrison *et al.* 1979). It has been suggested that these characteristics may reduce nutrient availability to the host animal (Leng, 1976; Bergen & Yokoyama, 1977) particularly on low-protein diets. Elimination of protozoa from the rumen has increased the growth rates and efficiency of food utilization for live-weight gain (Bird & Leng, 1978; Bird *et al.* 1979) indicating that under some circumstances the presence of protozoa in the rumen reduces animal productivity.

In cattle given sugar cane diets (Valdez *et al.* 1977) or grazing some temperate pastures (Clarke, 1965), although the small protozoa are present in the rumen, larger ciliates of the species *Isotricha* and *Dasytricha* are dominant. The volume of a large protozoan is

approximately 100 times that of a small protozoan and therefore, when the numbers of the two groups are equal, the large ciliates may represent 99% of the protozoal biomass. This biomass is often in excess of 5% of the volume of the rumen fluid (see Valdez *et al.* 1977) and may be over half the total microbial biomass (see Warner, 1962).

The experiments presented here are part of a continuing study of the involvement of protozoa in the nutrition of ruminants. These experiments were designed to examine the pool size and the rates of outflow of the large ciliate protozoa in relation to the turnover and outflow of the rumen fluid in cattle on sugar-cane-based diets and to obtain some indication of their importance relative to the bacterial pool.

Before studies in vivo were commenced, a number of preliminary experiments were undertaken. These included development of methods for isolating protozoa from rumen fluid and evaluating methods of measuring radioactivity in protozoa which had been labelled by incubation with [^{14}C]choline (see Coleman *et al.* 1980). Labelled protozoa were also incubated in rumen fluid in vitro to determine the rate of turnover of their ^{14}C-labelled constituents. The results indicated that there was a large pool of protozoa in the rumen of these cattle and that their turnover rate was slow in relation to the turnover of rumen fluid.

MATERIALS AND METHODS
Experimental animals and diets

Three Zebu bulls (350 kg live-weight) with permanent rumen cannulas were given free access to a diet of chopped whole sugar cane (intake 20–25 kg fresh material) and supplemented with 10 g urea/kg fresh cane. They were given 40 g/d of each of rock phosphate and sodium chloride and 750 g/d of wheat bran.

Rumen fluid sampling

Samples were taken by suction via a probe with a perforated end which was moved through a number of sites in the rumen as each sample was withdrawn.

Estimation of protozoal population density

Protozoa were counted in a standard counting chamber (0·2 mm depth) (Hawksley, Cristallite, Sussex, England) using rumen fluid diluted 1:5 with formal saline (10 g formalin in 90 g saline (9 g NaCl/l water)) as suspending medium.

Protozoa were differentiated into two groups. The first group were holotrichs and included *Isotricha* spp. with some *Dasytricha* spp. (large ciliates). The second group were oligotrichs mainly *Entodinia* spp. (small ciliates).

Uptake of [^{14}C]choline by protozoa incubated in rumen fluid

[^{14}C-Methyl]choline (5·5 μCi, 4 mg) (supplied by the Radiochemical Centre, Amersham, England) was added to 200 ml freshly-collected rumen contents in a conical flask and incubated at 39° for 20 h under nitrogen. Protozoal samples were taken from the flask periodically for the determination of radioactivity and N content. The radioactivity in cell-free rumen fluid was also obtained after high-speed centrifugation.

Estimation of the labelling of large and small ciliate protozoa

Protozoa, labelled as indicated, were isolated (see p. 373) and added to 200 ml freshly-collected rumen fluid. Following mixing, five subsamples of protozoa were isolated so as to contain varying numbers of large and small protozoa. This was accomplished by varying the time of sedimentation between washing.

Preparation of ^{14}C-labelled protozoa for in vivo and in vitro turnover studies
Rumen fluid (200 ml) was collected from each bull at 11.00 hours (approximately 1 h after feeding) and placed in a separatory funnel (excluding any air space) in an incubator at 39° for 1 h. In this time, protozoa settled and most of the plant material rose to the surface of the fluid. The protozoa and the 'plant-free' rumen fluid (approximately 130 ml) from each funnel were run into a conical flask containing [^{14}C-methyl]choline (1 mCi, 2 mg). The flask was gassed with N_2 and incubated in a water-bath at 39° for 2·5–3·0 h with shaking (80/min). The protozoa were then separated from the fluid by light centrifugation (500 *g* for 1 min) and the supernatant fluid discarded. Rumen fluid, which had been centrifuged at 25000 *g* for 20 min to remove plant material and micro-organisms, was used to resuspend the protozoa in the original fluid volume. The protozoal suspension was then lightly centrifuged as described previously and the supernatant fluid again discarded. Particle-free rumen fluid was again used to resuspend the protozoa. Two portions of 10 g of this suspension were taken (with care to ensure mixing): one for the estimation of total radioactivity in the protozoa and the other for studying the metabolism of ^{14}C-labelled compounds in protozoa in vitro (see p. 373). A 100 g portion of each washed ^{14}C-labelled protozoal suspension was injected into the rumen of the original donor.

Measurement of turnover of protozoa in rumen fluid incubated in vitro
Some of the labelled protozoal suspension (10 g) prepared as described previously was added to 200 ml rumen contents that had been collected 5 min earlier from the donor animal. The mixture was incubated at 39° for 20 h under N_2 with shaking (40/min). Samples (10 ml) were taken periodically for the isolation and estimation of radioactivity in protozoa.

Measurement of protozoa pool size and turnover in vivo
Labelled protozoa (100 g of the suspension in rumen fluid prepared as described previously) were injected into the rumen of the animal from which the protozoa had been originally obtained. Considerable care was taken to disperse the suspension throughout the rumen. Samples of rumen fluid were taken frequently over the first 30 h and thereafter once each day for 12 d, approximately 2 h after feeding. The large ciliates were isolated and assayed for N content and radioactivity.

Measurement of rumen volume and fluid outflow from the rumen
Polyethelene glycol (PEG; molecular weight 4000; 100 g) was injected intraruminally approximately 1·5 h before the injection of the suspension of labelled protozoa. Samples of rumen fluid were taken at intervals over 24 h and the concentration of PEG was estimated by the method of Malawar & Powell (1967). Rumen fluid volume and outflow rate were calculated assuming first-order kinetics.

Separation of protozoa for determination of specific radioactivity
Duplicate samples of rumen fluid (10 ml) were placed in test-tubes containing 1 ml glucose (10 g/l) and the mixture was incubated for 30 min at 39°. Plant particles rose to the surface leaving the protozoa almost free of plant debris. The protozoa were removed by withdrawing 2 ml fluid from the bottom of the tube using a syringe with a long needle and were immediately transferred to another tube containing 10 ml formal saline and allowed to stand for 30 min at room temperature. In this time the protozoa sedimented and any residual particulate matter either remained in suspension or sedimented on top of the protozoa. The supernatant fluid plus debris were removed by suction and the protozoa resuspended in 10 ml saline, centrifuged at 500 *g* for 2 min and the supernatant fraction

removed. The protozoa were washed in this way twice more (the final washings contained no residual radioactivity).

The protozoa were finally separated by centrifugation and the supernatant fraction discarded. Isotonic saline (4 ml) was then added and well mixed, and 4 portions (1 ml) were pipetted into scintillation vials. Protozoal numbers were determined on one portion; another was used for determination of N content; the remaining two portions were assayed for radioactivity.

Determination of radioactivity in protozoa

Protozoal suspensions (0·3–1·0 mg N in 1 ml saline) were initially assayed for radioactivity by three methods. In the first method the protozoa were oxidized (Van Slyke & Folch, 1940) and the carbon dioxide absorbed in CO_2-free sodium hydroxide and assayed for radioactivity as barium chloride (see Annison & White, 1961). In the second method the protozoa were solubilized by adding 10 ml of NCS™ (supplied by The Radioactivity Centre) to 1 ml protozoal suspension and heating at 40 ° for 12 h. A 5 ml portion of this solution was mixed with 10 ml scintillation cocktail and counted. In the third method, which was subsequently the method routinely used, the protozoa were suspended in 10 ml scintillation mixture containing toluene (670 ml), Triton X-100 (330 ml), 5 g PPO and 0·5 g POPOP and left for 24 h before assay in a liquid-scintillation spectrometer (Packard Tricarb Scintillation spectrometer 3255).

Determination of radioactivity in the dose of protozoa injected into the rumen

This was done essentially as indicated previously but since quantitative recovery of protozoa was necessary to determine the exact dose injected, the incubation period and the washing procedures were followed by light centrifugation (500 g for 2 min) to ensure total collection of protozoa (during the isolation and washing procedures).

Determination of radioactivity in rumen fluid and in bacteria

Rumen fluid (40 ml) was centrifuged at 25 000 g for 15 min, and 1 ml supernatant solution was placed in 10 ml scintillation mixture (see p. 374) and, following 24 h equilibration, assayed for radioactivity. Chemiluminescence occurred in these samples but had disappeared after less than 24 h in the cabinet of the scintillation counter.

The bacterial pellet remaining after high-speed centrifugation of rumen fluid was suspended in 40 ml isotonic saline and centrifuged at 25 000 g for 20 min. This process was repeated three times in order to remove extracellular radioactivity. The bacteria in the pellet were finally suspended in 5 ml isotonic saline and 1 ml mixed bacterial suspension was assayed for radioactivity as indicated for protozoal preparations.

Background counts in liquid-scintillation counting

The background count rates in the systems used for assay of radioactivity in rumen protozoa, bacteria and fluid were determined by isolating the various preparations from rumen fluid collected from each animal the day before injection of labelled protozoa, and counting under identical conditions to those of labelled samples.

Chemical methods

VFA concentrations and proportions. VFA proportions were determined by gas–liquid chromatography using the column and conditions recommended by Erwin *et al.* (1961). The concentrations of VFA were determined using isovaleric acid as an internal standard. This standard was selected after initial examination had indicated there was no detectable isovaleric acid in the rumen fluid of these animals. Rumen fluid (1 ml) plus 0·1 ml of an isovaleric acid standard (2·01 g/100 ml) were placed in a tube, centrifuged at 1000 g for

Table 1. *Liquid-scintillation counting of protozoa ($\mu Ci/g$ protozoal N) in various systems*

(Protozoa were isolated from 50 ml rumen fluid, made up to a volume of 10 ml with isotonic saline and 1 ml was taken for estimation of N and for counting (see p.374))

Assay system	A*	B	C	D
BaCO₃ in toluene, PPO, POPOP	2·18	2·51	—	—
NCS™/Triton X-100/toluene, PPO, POPOP	2·20	2·38	155	175
Triton X-100 toluene, PPO, POPOP	2·15	2·42	160	180

* Samples A and B were obtained from the rumen of animals and C and D from an in vitro incubation of protozoa in rumen fluid with [¹⁴C]choline.

15 min and 4 μl supernatant fraction was injected into the column. Recoveries of isovaleric acid were used to correct the other VFA peaks for quantity of sample injected. The concentration of each acid was calculated using a standard curve of peak height *v.* concentration for each acid.

Concentration of ammonia in rumen fluid. Ammonia from rumen fluid was isolated by steam distillation as described by Nolan & Leng (1972) except that saturated sodium tetraborate (10 ml) was used as the alkaline reagent.

Total N. Protozoal suspension (1 ml) was oxidized by standard Kjeldahl techniques and the digest mixture distilled and titrated as described for ammonia.

Dry matter content of rumen protozoa. Protozoal samples were isolated from 100 ml rumen fluid as described previously and suspended in 5 ml saline. A portion (1 ml) was assayed for total N content; a 2 ml sample was weighed and dried in a tared scintillation vial and reweighed after drying at 95 ° for 2 d in order to estimate dry matter content.

RESULTS
Assay of radioactivity in protozoa
When protozoa, (containing 0·2–0·5 mg N) in 1 ml isotonic saline, were mixed with the scintillation mixture for a period of 8 h, all the radioactivity was apparently extracted into the mixture, since the count rate of scintillation mixture was unchanged by excluding the protozoal cells immediately before liquid-scintillation counting (by decantation) or resuspending them in the scintillation cocktail.

The radioactivities of samples of protozoa determined by three methods were not different (Table 1). Using the third method, the coefficient of variation of the estimate of specific radioactivity of protozoa for five samples isolated from one rumen fluid sample was 8·9%.

The effects of the concentration of protozoa on efficiency of scintillation counting
A sample of labelled protozoa was isolated from the rumen fluid of one animal and diluted in isotonic saline to give $1·7–8·0 \times 10^4$ large ciliate protozoa/ml (approximately 0·1–0·5 mg N). Each sample was assayed for radioactivity by mixing it in the scintillation mixture and counting after 24 h. There was a linear relationship between the number of protozoa and radioactivity in the sample (Fig. 1) indicating that the concentration of protozoa (over the range assayed in these studies) had no detectable effect on the efficiency of scintillation counting.

Differential labelling of protozoa
The N content and specific radioactivity of five different preparations of protozoa from a sample of rumen fluid which contained ¹⁴C-labelled protozoa are given in Table 2. There
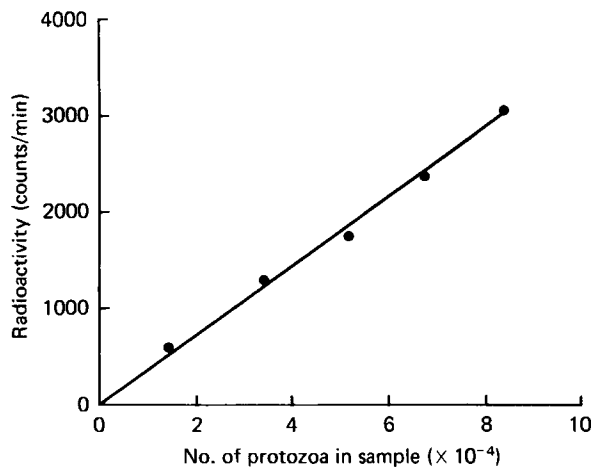
Fig. 1. The relationship between the measurement of radioactivity and of number of protozoa present in different dilutions of a standard sample.

was a highly significant ($P < 0.001$) relationship between numbers of large ciliates (H; $\times 10^{-4}$/ml) and the radioactivity (R; counts/min) in the sample (n 5), i.e.

$$R = 1047\ (\pm 94)\ H, \quad r^2\ 0.97. \tag{1}$$

Inclusion of the numbers of small ciliates in a multiple regression did not remove a significant additional part of the variation.

Similarly there was a highly significant ($P < 0.001$) relationship between N content (N_c; mg N/sample) and numbers of large ciliates in the sample (Fig. 2). This was also not significantly affected by the number of small ciliates present.

$$N_c = 0.05\ (\pm 0.004)\ H + 0.08, \quad r^2\ 0.85. \tag{2}$$

Protozoal preparation

The method for preparing ^{14}C-labelled protozoa for injection and also for isolating protozoa for determination of radioactivity resulted in a decrease in the ratio of numbers of small to large ciliate protozoa. This value was 8:1 in the animals shortly after feeding. However in the labelled dose preparations and in the isolated samples taken for determination of radioactivity the value was approximately 1:1.

The relationship (Fig. 2) indicates that 1.8×10^5 large ciliate protozoa contained approximately 1 mg N and analysis of dried protozoal samples indicated that these protozoa (i.e. in the fed state) contained 28 g N/kg dry matter. The intercept value indicates some N was present in the samples which was not associated with the large ciliates. Some small ciliates (*Entodinia* and *Epidinia* spp.) were present in the samples counted; small quantities of other nitrogenous materials in the sample or small errors in the protozoal counting or N determinations may also have contributed to the intercept. Multiple regression analysis which included the numbers of small ciliate protozoa did not significantly improve the relationship.

Dynamics of fluid in the rumen

Rumen fluid volume, half time ($t_\frac{1}{2}$) and the rate of flow of fluid out of the rumen of the three animals measured on the first day following injection of labelled protozoa are given in Table 3.

Table 2. *Effect of the presence of small ciliate protozoa on the estimation of the specific radioactivity of the large ciliate protozoa*

Sample no.	Protozoal nos. ($\times 10^{-4}$/ml)		Nos. large ciliates:nos. small ciliates	Sample N content (mg N/ml) (A)	Adjusted* N content (mg N/ml) ($A \times 3\cdot2/x$)	Radioactivity in sample (counts/min per ml) (B)	Adjusted* radioactivity in sample (counts/min per ml) ($B \times 3\cdot2/x$)
	Large ciliates	Small ciliates					
1	3·2	4·2	0·76	0·179	0·179	2903	2903
2	2·2	1·2	1·83	0·164	0·239	2237	3254
3	1·9	5·2	0·36	0·152	0·221	2372	3995
4	1·5	0·8	1·88	0·106	0·226	2216	3732
5	1·1	3·1	0·35	0·082	0·238	1040	3025

* Adjusted to the same number of large ciliates as in sample no. 1 (i.e. $3\cdot2 \times 10^4$). x represents the no. of large ciliates in each sample.
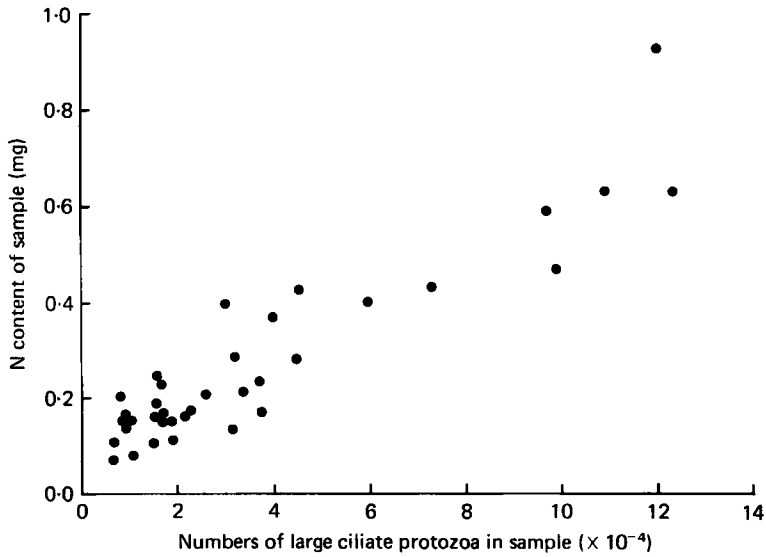
Fig. 2. The relationship between the content of protozoal nitrogen and the number of large ciliate protozoa in a sample.

Table 3. *Dynamics of fluid in the rumen of cattle given sugar cane based diets*

	Rumen fluid			
Animal no.	Volume (l)	Half-time (d)	Outflow rate (1/d)	Dilution rate (volumes/d)
93	62	0·39	109	1·8
94	48	0·26	125	2·6
95	53	0·32	114	2·2
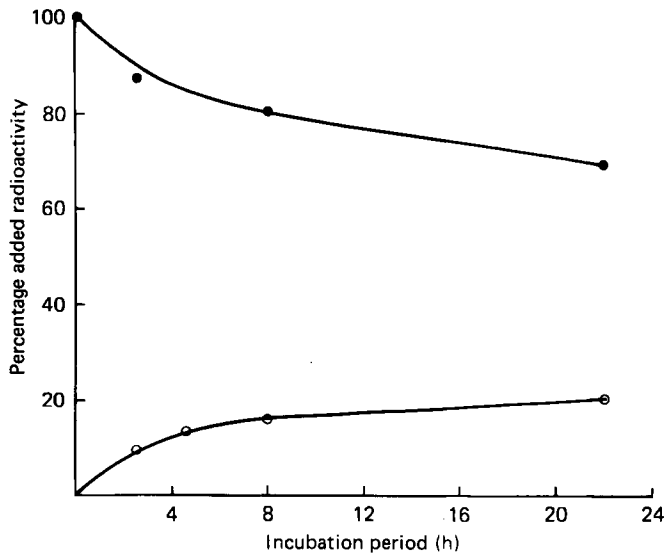
Fig. 3. The rate of incorporation of radioactivity in protozoa incubated in rumen fluid containing [^{14}C-methyl]choline. (●), In solution; (○), in protozoa.
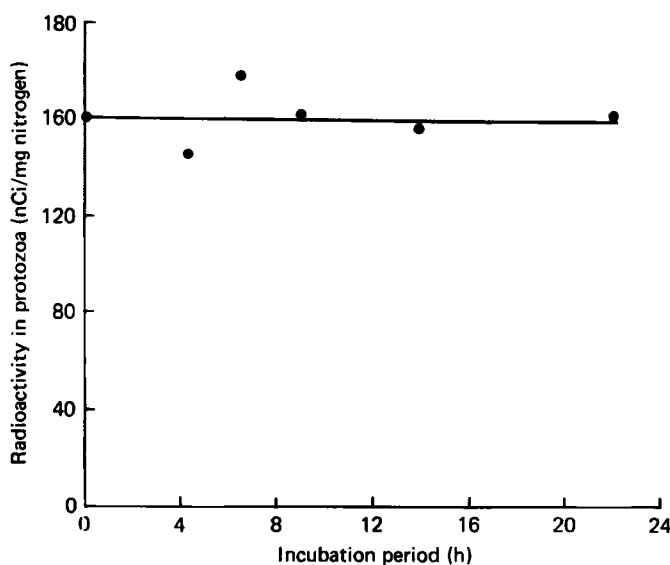
Fig. 4. Turnover of ^{14}C-labelled protozoa in vitro. The relationship between radioactivity in protozoa and time of incubation in vitro following preparation of ^{14}C-labelled protozoa.

The uptake of choline by protozoa in rumen fluid in vitro

Protozoa appeared to take up choline over a 20 h period; 10% of the radioactivity added to the flasks was incorporated into the protozoa within 2·5 h, increasing to over 20% after 20 h. The ^{14}C in solution declined only slowly. The majority of the radioactivity not in solution could be accounted for in the protozoa (see Fig. 3).

The specific radioactivity of labelled protozoa incubated in rumen fluid

When 10 ml of the labelled protozoal preparation (the same as that injected into the rumen) was incubated for 20 h in 200 ml freshly-drawn rumen fluid, there was no detectable decrease in the specific radioactivity (nCi/mg N) of protozoa (Fig. 4).

The turnover of protozoa in the rumen of the animal

The SR of protozoa (μCi/g N) over a period of 13 d following a single intraruminal injection of ^{14}C-labelled protozoa is given in Fig. 5. After initial mixing there was only a slow rate of decrease of SR over a period of 5 d but this rate apparently increased between 5 and 13 d (see Fig. 5). The kinetics of protozoa (Table 4) were calculated from the decline in SR of protozoa between 0 and 5 d assuming steady-state conditions in the protozoal pool.

No radioactivity was detected in a sample of cell-free rumen fluid or in rumen bacteria obtained 12 h after the injection.

Rumen fluid VFA and ammonia concentrations and protozoal numbers

The mean concentrations of VFA over 24 h were in excess of 100 mmol/l; immediately before the morning feeding they were approximately 80 mmol/l. In general, after feeding, acetate concentrations decreased and propionate concentrations increased. Immediately before the morning feed the proportions of acetate, propionate and butyrate were 72, 18, 10 respectively. These changed to 66, 24, 10 about 4 h after feeding and then slowly returned to the prefeeding values. Ammonia concentrations in rumen fluid followed a diurnal pattern reaching 12 mmol/l during feeding and then falling to 2–5 mmol immediately before feeding.
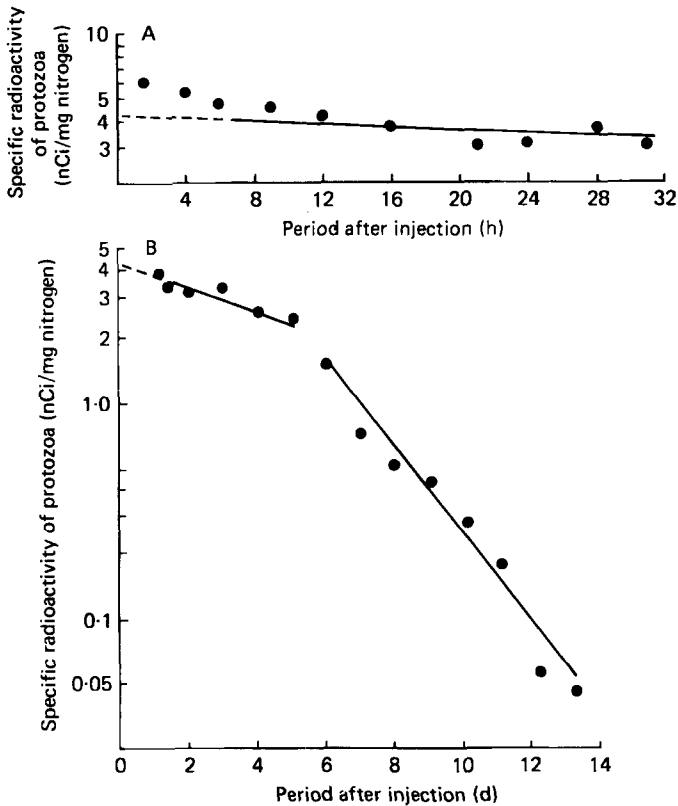
Fig. 5. The relationship between specific radioactivity of protozoa (nCi/mg nitrogen) and time after injection of ^{14}C-labelled protozoa into the rumen of a 350 kg bull given a sugar-cane-based diet. (A), 0–32 h; (B), 0–13 d. The line of best fit for up to 5 d is shown on both plots.

Table 4. *Dynamics of protozoa (large ciliates) in the rumen of cattle given sugar cane based diets estimated by single injection of ^{14}C-labelled protozoa into the rumen*

| | Protozoal no. in rumen fluid ($\times 10^{-4}$) | | | | Pool* size (g N) | Half-time (d; $t_{\frac{1}{2}}$) | | Apparent irreversible loss rate at 0–5 d† (g N/d) |
| Animal no. | Before feeding | | After feeding | | | | | |
	L	S	L	S		0–5	5–13	
93	0·8	18	4·0	31	46	14·3	2·1	2·2
94	0·6	15	3·3	21	26	5·7	1·4	3·2
95	0·4	17	2·6	18	24	5·4	1·8	3·1

L, holotrichs (80% *Isotricha* and 20% *Dasytricha* spp.); S, oligotrichs (mainly *Entodinia* spp.)

* Pool size (P_o) was calculated from the specific radioactivity (SR) *v.* time relationship over 5 d, i.e. $P_o = I/SR_o$, where SR_o is the specific radioactivity of isolated protozoa (μCi/g N) at zero time and I is the injected dose (μCi) of radioactivity in protozoa.

† Calculated as $P_o/t_{\frac{1}{2}} \times 0·693$.

Protozoal numbers in rumen samples taken over 24 h followed a characteristic diurnal pattern (see Valdez *et al.* 1977). The mean numbers of protozoa in samples of rumen fluid taken at hourly intervals from 4 h before feeding to 4 h after feeding are shown in Table 4.

DISCUSSION
Methodology

A necessary part of this study was the development of a simple but accurate method for determination of the radioactivity in protozoa. In initial studies the amount of radioactivity in a standard sample of protozoa determined by three methods was found to be similar and subsequently the protozoa were assayed in a solvent mixture which appeared to extract quantitatively the labelled components. The efficiency of liquid-scintillation counting was high and unaffected by chemiluminescence, quenching or self-absorption by the cell debris within the range of protozoal numbers in the isolated samples.

The three animals used in these studies had large populations of protozoa in the rumen consisting of approximately 5×10^4 large ciliates/ml (approximately 80% *Isotricha* spp. and 20% *Dasytricha* spp.) and approximately 4×10^5/ml small ciliates, mainly *Entodinia*. These concentrations indicate that the large ciliates were approximately 90% of the protozoal biomass in rumen fluid (calculated according to Warner's (1962) information on the relative volumes of protozoa; see Leng, 1976). However in the labelled protozoal preparation or in samples of isolated protozoa the large ciliates were further concentrated such that the small ciliates represented only 1–2% of the final volume of protozoa. The small ciliates were therefore unlikely to have represented an appreciable fraction of the N content of isolated preparations. This conclusion is supported by the relationship given in Fig. 2 which indicates that the large ciliates accounted for at least 90% of the total N in any sample.

During the preparation of labelled protozoa, it was likely that all species of protozoa incorporated [^{14}C]choline. If the small ciliates became highly labelled relative to the large ciliates their presence would have biased the results. However, by preparing, from a single sample of rumen fluid, a number of protozoal samples with a range of large to small ciliate numbers and assaying the samples for radioactivity, it was found that the small ciliates made little contribution to the total radioactivity of any isolated protozoal sample (Table 2).

Neill *et al.* (1979) demonstrated a considerable loss of radioactivity from solution when [^{14}C]choline was incubated with rumen fluid from sheep. This is in contrast to the present studies where the loss of radioactivity from rumen contents was relatively small. It is possible that choline was metabolized and that trimethylamine (a precursor of methane and the main route of loss of radioactivity from [^{14}C-methyl]choline) accumulated in rumen fluid from these cattle as occurred in rumen fluid from fed sheep (Neill *et al.* 1978). Protozoa incorporated 10% of added radioactivity as [^{14}C]choline in a 2 h incubation.

Pool size of protozoa

As shown by Valdez *et al.* (1977) and Minor *et al.* (1977) the numbers of protozoa present in samples of rumen fluid extracted from cattle on this diet apparently varied with time after feeding. The numbers of protozoa in a sample, however, underestimate the concentrations in the rumen (Minor *et al.* 1977). Calculation of pool size of protozoa in the rumen by multiplying fluid pool size by protozoal N predicted from protozoal counts in rumen fluid samples using the relationship shown in Fig. 2 is therefore subject to considerable error.

Following injection of the labelled protozoa into the rumen the radioactivity per unit N in protozoa decreased only slowly over the first 5 d. Assuming that first order processes occurred, the protozoal N pool size can be calculated from the zero-time intercept of the specific radioactivity-time relationship (Fig. 5). The pool size of protozoal N (calculated

from tracer dilution) was 24–46 g N. The pool size of protozoal N calculated from the relationship given in Fig. 2 and the fluid volumes in the rumen, was always less than that calculated from the tracer studies. Estimates made using the highest numbers of holotrich protozoa recorded, which were always shortly after feeding, indicate that between 60 and 90% of the total protozoal population within the rumen were available to be sampled. In rumen fluid samples taken 12–24 h after feeding only 5–20% of the protozoal population was present in the sampled fluid pool. These results support the conclusion of Clarke (1965) and Minor *et al.* (1977) that the total population of protozoa in the rumen cannot be simply estimated by multiplying numbers of protozoa per ml by rumen volume.

Turnover of protozoa

The apparent turnover of labelled carbon in protozoa in steady state in the rumen is a function of: (1) division and synthesis of new protozoal cells which is equal to the death of protozoa in the rumen plus the passage of protozoa to the lower digestive tract; (2) the irreversible release into the medium of compounds, originally labelled by [14C]choline. Without estimates of 14C-labelled protozoa entering the lower digestive tract the apparent turnover of protozoa in the rumen cannot be partitioned between the relative contributions of protozoal outflow and protozoal lysis.

Following injection and mixing of the labelled protozoa in the rumen pool the decrease in SR of protozoa (μCi/mg N) over the first day was not detectable even though a relatively large number of samples were taken at frequent intervals for up to 30 h (see Fig. 5). Thereafter SR of protozoa decreased only slowly over the first 5 d. However, the mean apparent disappearance rate of protozoa, was only 2–3 g N/d (see Table 4), and this includes both lysis and passage to the lower tract. There was apparently little 14C released from protozoa in a 20 h in vitro incubation indicating that the 14C lost from living protozoa was small. If this is also the situation in the rumen, then the turnover in the rumen is almost entirely due to passage of protozoa down the tract. However, in all three animals the apparent turnover of the protozoal pool was less than 0·2/d, indicating very slow movement of protozoa out of the rumen relative to the rumen fluid turnover which was 1·8–2·6/d.

Although the large ciliates in the rumen on this diet constituted a large biomass (16–24 mg DM/ml), they apparently contribute only a small proportion of the total microbial protein synthesis in the rumen. An estimate of the net microbial protein synthesis in the rumen of the cattle used here can be obtained by using the lowest value for microbial protein leaving the rumen of cattle on similar diets reported Elliott *et al.* (1978) (i.e. 30 g N/kg organic matter digested in the rumen). Using this value some 800 g microbial protein may have become available for digestion in the intestines in the animals used here whereas only approximately 30 g protozoal protein was apparently synthesized daily. Thus protozoal protein synthesis appears to represent a negligible proportion of the total microbial protein synthesized in the rumen of these cattle.

On approximately the fifth day of the experiment the rate of decline of SR of protozoa apparently increased substantially in all animals. The apparent turnover of the protozoal pool increased from less than 0·2 to 0·5–0·7/d (Fig. 5). This was still substantially less than the rate of flow of fluid out of the rumen but the increase is indicative of a much greater disappearance rate of the labelled protozoa from the rumen pool. The reasons for the increased rate of loss of labelled protozoa are not clear. It is possible that only those protozoa that were actively dividing incorporated most of the [14C]choline and subsequently had a life span in the rumen of approximately 5 d before dying and being fermented or washed from the rumen. There were no obvious changes in the diet and feeding pattern of the animals and no apparent changes in the protozoal numbers in the rumen over the 13 d period.

Much of the radioactivity in protozoa after incubation with [^{14}C]choline is probably in the form of phosphocholine (Bygrave & Dawson, 1976) which is slowly used to synthesize phosphatidylcholine as this is turned over in protozoal tissues. It is possible that initial turnover rate is influenced by the combined turnover of the phosphocholine and phosphatidylcholine pools. Once the reserves of the former are reduced then the turnover of the phosphatidylcholine pool may be the dominant influence on the SR of protozoa which in turn would be influenced by the uptake and turnover of unlabelled choline from rumen fluid.

Singh *et al.* (1974) attempted to estimate turnover of protozoa in the rumen of buffaloes using protozoa labelled by incubation for 12 h with [^{14}C]glucose. In these studies the $t_{\frac{1}{2}}$ of protozoal carbon (species and numbers present were not reported) was approximately 1000 min. However the carbon components of protozoa that were labelled and the extent of their turnover during protozoal metabolism were not determined. Ulbrich & Scholz (1966) obtained an estimate of $t_{\frac{1}{2}}$ for N in large ciliate protozoa in the rumen of lactating cows from the decline in enrichment of protozoal N after removal of [^{15}N]urea from the diet. The $t_{\frac{1}{2}}$ was 24–48 h. This $t_{\frac{1}{2}}$ for ^{15}N is not comparable with the $t_{\frac{1}{2}}$ values obtained in the present study because ^{15}N is labile and a decline in enrichment occurs as a result of normal metabolism of protozoa living in the rumen as well as by their loss and replacement with new unlabelled protozoa. In contrast, protozoa labelled with [^{14}C]choline had almost constant SR during a 24 h incubation in vitro, suggesting that the decline in SR in vivo was probably due only to their loss and replacement.

The important new insight from these studies is that the growth rate of large ciliate protozoa (when these constitute a large biomass in the rumen) is small relative to the calculated growth rate of bacteria. The importance of large ciliates in the digestive physiology of ruminants may therefore result from their physical presence in the rumen which may reduce the biomass of bacteria. Although their growth rate is relatively low, their selective retention within the rumen means that their biomass may at times exceed that of bacteria. The potential detrimental effect to the animal of a substantial biomass of large ciliates may be a result of competition for substrate and engulfment and digestion of bacterial protein by protozoa, resulting in decreases in the bacterial biomass and flow of protein and energy from the rumen. Low growth rates of these protozoa would still be associated with high rates of substrate use; the energy available to the organisms as ATP being used for maintenance and their high mobility (activity).

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