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Dynamics of protozoa in the rumen of cattle

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1. The dynamics of protozoa were studied in two groups of rumen-fistulated cattle fed on a basal diet of molasses *ad lib.*, with oaten chaff given at 6 or 18 g/kg live weight. This diet resulted in different mixtures of protozoal species in the populations in the rumen.

2. The rumen protozoa were studied by intrarumen injections of protozoa labelled in vitro with [$^{14}CH_3$]choline. An indication of protozoal death and fermentation of protozoal cell residues was obtained by measuring ^{14}C loss via the methane pool.

3. After a single injection of labelled protozoa, the decline in the specific radioactivity (μCi/g nitrogen) of the protozoal pool in the rumen indicated that first-order kinetic processes applied. Conversely the specific radioactivity of protozoa, incubated in rumen fluid, remained constant indicating no growth in vitro, presumably owing to a rapid exhaustion of essential nutrients.

4. The protozoal populations in the rumen of cattle fed on the diet with the low level of oaten chaff were mainly small ciliates; but on the higher level of chaff in the diet, the large ciliates were a higher proportion of the total protozoal population present.

5. The mean pool size of protozoa in the rumen was significantly larger and the protozoal half-life tended to be longer for cattle fed on the higher level of chaff in the diet. The apparent production rate of protozoa in cattle fed on each diet was not significantly different and there were no differences in the production rate of methane. The percentage losses of label from protozoa in the rumen via the methane pool were not significantly different on the two diets and indicated that 74% of the protozoa that were apparently irreversibly lost from the rumen could be accounted for by death and lysis in the rumen and therefore only 26% of protozoa apparently entered the lower digestive tract.

The influence of protozoa in the rumen on the nutrition of ruminants has been investigated in recent years. There is now considerable information that indicates that the presence of a large population density of protozoa in the rumen decreases the amount of microbial and dietary protein that becomes available for digestion in the small intestine (see Bird & Leng, 1978*a*, *b*, 1985; Bird *et al.* 1979; Demeyer & Van Nevel, 1979; Veira *et al.* 1984; Veira, 1986; Ushida *et al.* 1986).

It is possible to produce viable labelled protozoa by incubating rumen fluid containing protozoa with [$^{14}CH_3$]choline and to maintain them viable before injection into the rumen (Coleman *et al.* 1980; Leng, *et al.* 1981; Leng, 1982). The re-introduction of the labelled protozoa and subsequent decline in their specific radioactivity allows estimates to be made of their pool size and turnover rate in sheep (Leng, 1982) and cattle (Leng *et al.* 1981, 1986). The studies to the present time in cattle have been confined to three adult animals fed on a diet of sugar cane and wheat bran (Leng *et al.* 1981) and six mature cows fed on cut green pasture where the protozoa were mainly large holotrichs (i.e. *Isotricha* and *Dasytricha* spp.; Leng *et al.* 1986). The studies now presented were undertaken in order to provide information on the dynamics of the small protozoal species in the rumen of cattle fed on two diets of *ad lib.* molasses and restricted forage. These protozoa are more representative of the protozoal populations that are present in the rumen of cattle and sheep fed under intensive production systems.

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The results confirm the retention of small protozoa in the rumen but there is a suggestion of an effect of liquid turnover rate on protozoal populations present. The production rate of protozoa was the same on both diets.

MATERIAL AND METHODS

Experimental animals and diets

Four Hereford steers (286 kg) and five heifers (239 kg) were each fitted with a rumen cannula at least 8 weeks before the experimental period. During this time, molasses (containing 30 g urea/kg) was continuously available to all animals and oaten chaff was offered once daily (at either 300 or 700 g chaff/kg diet). The steers received 8 g chaff (on a dry matter (DM) basis)/kg live weight (LWt) and, in a subsequent period, heifers received 16 g chaff DM/kg LWt. Mean DM intakes (molasses + oaten chaff) by steers and heifers were 6·0 (SD 0·15) and 5·1 (SD 0·67) kg/d respectively.

Experiment

A series of repeated studies were undertaken to measure the dynamics of rumen protozoa in cattle fed on the two diets (low and high levels of forage), using intrarumen injections of ^{14}C-labelled protozoa and CrEDTA to measure protozoal and fluid dynamics. At 2 or 3 d following the measurement of protozoal dynamics the rate of methane production was measured using ^{14}C-labelled methane (Murray *et al.* 1976).

Experimental procedures

Preparation of labelled protozoa. The preparation of labelled protozoa was a scaled-up modification of the method described by Leng (1982). Briefly, rumen fluid (250 ml) was collected into a warmed glass syringe by gentle suction through a probe situated in the rumen. The fluid was transferred to a conical flask in a water-bath at 39° and incubated with [$^{14}CH_3$]choline (obtained from Amersham International plc, Amersham, Bucks) for 2 h. Where numbers of protozoa in the rumen fluid were low, the protozoa were first concentrated by centrifuging at 500 ***g*** for 1 min and then a portion of the supernatant fraction was removed. The remaining fraction was remixed using a vortex mixer and then incubated for 2 h. The ^{14}C-labelled protozoa were isolated by centrifugation, washed with rumen fluid (collected a few minutes before from the donor animal), centrifuged again and finally suspended again in fresh rumen fluid before being injected into the rumen of the animal from which they had been obtained originally.

Isotope studies of the dynamics of protozoa in vivo. Following injection of labelled protozoa, samples of rumen fluid (40 ml) were taken at intervals of approximately 3 h over a period of up to 3 d. Protozoa were isolated and assayed for N content and radioactivity as previously described (Leng, 1982). Rumen fluid samples were analysed for ammonia, volatile fatty acid (VFA) concentrations and proportions, and protozoa were enumerated (see Leng, 1982). Gas samples were taken every 3 h from the gas space in the rumen for determination of the specific radioactivity of the methane.

Estimation of protozoal turnover and lysis in vitro. This was performed to check previously reported results with sheep that indicated no loss of ^{14}C relative to N from the labelled protozoa in rumen fluid. Two approaches were used in a series of experiments. In the first study, labelled protozoa were prepared as described previously and injected into freshly collected rumen fluid held in a conical flask under carbon dioxide and incubated at 39° with occasional shaking (flask 1). In the second approach, labelled protozoa were injected into the rumen of cattle and 26 h later approximately 1 litre rumen fluid was obtained and incubated under the same in vitro conditions (flask 2). Samples of rumen fluid were taken

from these flasks at frequent intervals over a period of 1400 min. Protozoa were isolated and assayed for radioactivity. In some experiments, gas was also collected from the space above the rumen fluid to determine the radioactivity in methane.

Assay of specific radioactivity of methane gas. The specific radioactivity of methane in rumen gas was determined as described previously (Leng, 1982).

Calculations

Protozoal dynamics. The specific radioactivity in protozoa (μCi/g N; SR) with time-interval following injection of ^{14}C-labelled protozoa was described by a single exponential function of the form:

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

Where SR_t and SR_0 are specific radioactivities at time t and 0 respectively, and m is the rate constant (slope $\times$ 1·44).

Pool size (P_0, g N) was calculated as:

$$P_0 = \frac{I}{SR_0},$$

where I (μCi) is the injected dose.

The apparent production rate (g N/d; A) of protozoa was calculated from

$$A = P_0 m.$$

Protozoal lysis rate. The specific radioactivity of methane with time was also described by a single exponential function. The area under this relation ($SR_0 \times (1/m)$) multiplied by methane irreversible loss rate (measured in a separate experiment using a continuous infusion of [^{14}C]methane) was taken to be the total radioactivity lost via the methane pool. The ratio radioactivity lost via methane:radioactivity in the injected dose was taken to represent the proportion of the protozoal pool lysed in the rumen (see Leng, 1982).

Rumen fluid dynamics. Rumen fluid volume, half-life and outflow rate of fluid were calculated by standard techniques from the decline with time in concentrations of CrEDTA and [^{15}Cr]EDTA in the rumen fluid after single injections of these markers (Downes & McDonald, 1964).

Statistics

Period effects between studies were expected to be small compared with the effect of forage level because there was little variation of the environmental condition in the housing unit throughout the experimental periods. The main effect of sex is manifested primarily in LWt and feed intake, thus steer and heifer comparisons in terms of the effect of forage level in the molasses-based diet were tested after making adjustments for LWt and DM intake by analysis of co-variance.

RESULTS

Experimental animals. In the first of the two studies with steers, which received the low level of the forage/molasses diet, observations of rumen-fluid kinetics and protozoal dynamics were obtained from all four animals. When the study was repeated 30 d later, observations on rumen fluid and protozoal dynamics were only obtained in two and in three of the animals respectively because the other animals showed symptoms of molasses toxicity. For similar reasons, only eight observations were made of the dynamics of rumen fluid and seven of the dynamics of protozoa in the heifers. Where observations were duplicated in one animal, the mean value was used in comparisons between experimental diets.

Fluid dynamics and metabolites. The pool size, half-life and rate of outflow of rumen fluid together with the concentrations of rumen ammonia for the two groups of cattle are given in Table 1. The mean rumen fluid concentration of VFAs in cattle fed on the high-forage

Table 1. *Rumen fluid kinetics and rumen ammonia concentration in cattle fed* ad lib. *on a molasses-based diet supplemented with oaten chaff at either 8 or 16 g/kg live weight (LWt)*

(Mean values with their standard errors)

			Rumen fluid									
			Volume* (l)		Half-life* (min)		Outflow rate* (l/d)		Fractional outflow rate* (%/h)		Ammonia concentration (mg nitrogen/l)	
Animals	*n*	Dietary forage (g/kg LWt)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Steers	4	8	40	2·5	600	26	64	2·7	7·1	0·76	165	29
Heifers	5	16	25	2·1	422	23	60	2·3	10·2	0·66	100	26

* Adjusted to same LWt and dry matter intake by analysis of covariance.

diet was 93 (SE 2·2) mM and the molar proportions of acetic, propionic and butyric acids were 66, 20 and 12 respectively. Results for VFAs in the rumen of cattle fed on the low-forage diet are not available.

Rumen-fluid volumes were smaller and the fractional outflow rates of rumen fluid were higher in the cattle fed on the high-forage diet, but outflow rate was not significantly different between the cattle fed on the two diets.

Protozoal species. Entodinium spp. (20 μm) were exclusively found in the rumen of two of the animals given the low-forage diet. The other two animals had predominantly *Entodinium* spp. with *Epidinium* spp. (40 μm) or *Polyplastron* spp. (150–200 μm) comprising about 1% of the total protozoal numbers. In the rumens of animals fed on a high level of forage, the *Entodinium* spp. were predominant and *Epidinium* spp. ranged from 3 to 17% of the total protozoal numbers (a few *Polyplastron* spp. were also present) and about 18% were represented by large *Isotricha* spp. (60 μm). The remainder of the protozoal population consisted of entodinia and diplodinia (30 μm) species.

Apparent production rate and 'lysis rate' of protozoa in vitro. When ^{14}C-labelled protozoa were incubated in rumen fluid in vitro there was little change in specific radioactivity of isolated protozoa over 24 h (regression coefficients for each flask were not significantly different from zero; see Fig. 1). Where labelled protozoa were injected into the rumen of an animal and rumen fluid was collected about 26 h after the injection and incubated in a conical flask at 39° for 10 h, the specific radioactivity of the isolated protozoa also remained constant. In contrast, the specific radioactivity of protozoa in the rumen of the animal over the same period continued to decline according to a first-order kinetic process. The results for both in vitro and in vivo studies are shown in Fig. 2.

The specific radioactivity of methane collected from the in vitro incubation was higher than that of the methane collected from the gas space in the rumen of the animal (Fig. 2).

Measurement of dynamics of protozoa in the rumen. The changes in specific radioactivity of protozoa and methane following an intrarumen injection of labelled protozoa are also shown in Fig. 2. The estimates of pool size, half-life and apparent production rate of protozoa are given in Table 2, with the mean and range of estimated protozoal population densities in rumen samples taken during each experiment. The estimated rate of methane production, and of the percentage of the radioactivity in injected ^{14}C-labelled protozoa that passed through the methane pool are also shown in Table 2.

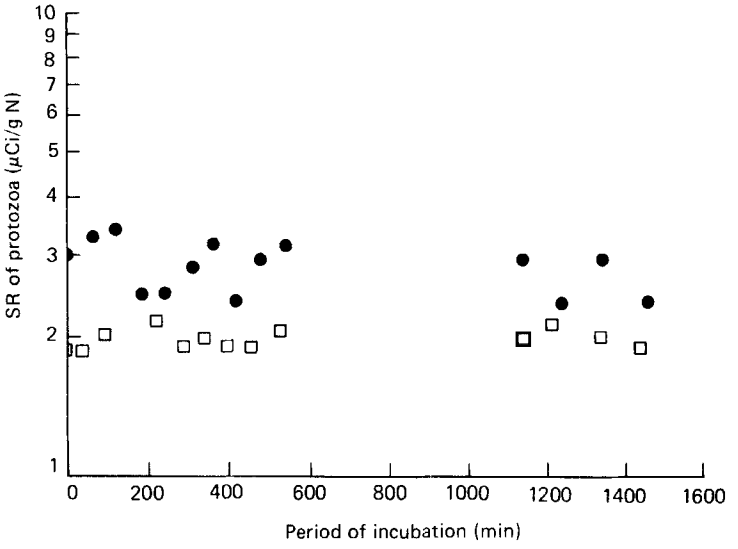

Fig. 1. The specific radioactivity (μCi/g nitrogen; SR) with time-interval (min) of protozoa incubated in rumen fluid in vitro at 39° following labelling of protozoa with [$^{14}CH_3$]choline. (●), Flask 1; (□), flask 2. For details of procedures, see p. 430.

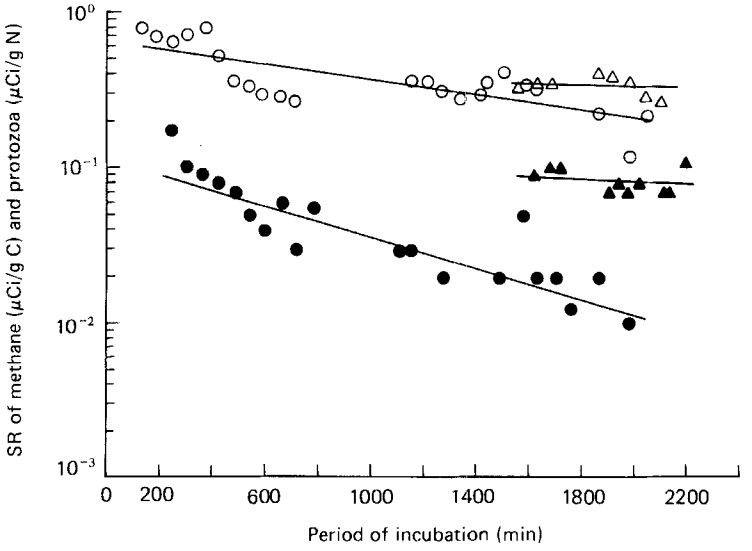

Fig. 2. The specific radioactivity (μCi/g nitrogen; SR) of protozoa isolated from the rumen following a single injection of ^{14}C-labelled protozoa (○). At 26 h after the injection, 1 litre rumen fluid was incubated in vitro at 39° and the SR of protozoa (△) was determined at intervals of about 80 min. The SR of methane in the rumen gas samples (●) or in gas samples collected from the in vitro incubation (▲) are represented by the two lower lines.

Regression equations:

SR of protozoa:

in vivo $\quad \mathrm{Log}_n y = -0{\cdot}00057x - 0{\cdot}42 \quad R^2$ 0·59, RMS 0·30,
in vitro $\quad \mathrm{Log}_n y = -0{\cdot}00009x - 0{\cdot}89 \quad R^2$ 0·02, RMS 0·15,

SR of methane:

in vivo $\quad \mathrm{Log}_n y = -0{\cdot}00102x - 2{\cdot}19 \quad R^2$ 0·76, RMS 0.37,
in vitro $\quad \mathrm{Log}_n y = -0{\cdot}003x - 1{\cdot}90 \quad R^2$ 0·12, RMS 0·17,

where RMS is the residual mean square.

Table 2. *The dynamics of protozoa in the rumen of cattle fed* ad lib. *on a molasses-based diet supplemented with oaten chaff at either 8 or 16 g/kg live weight (LWt)*

					Protozoa									
		Dietary forage (g/kg	No. of Protozoa ($\times 10^{-5}$/ml)		Pool size* (g nitrogen)		Half-life* (min)		Production* rate (g N/d)		Methane production* (g carbon/d)		Percentage injected ^{14}C lost in methane	
Animals	*n*	LWt)	Mean	Range	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Steers	4	8	7·0	2·4–10·4	16	1·7	929	38	17	2·7	56	7·6	80	15
Heifers	4	16	3·12†	0·4–6·8	24	1·4	1066	38	21	2·3	48	7·6	68	15

* Adjusted to same LWt and dry matter intake by analysis of covariance.
† The ratio, obligotrichs:holotrichs was 2·8:0·32 in the mean numbers.

DISCUSSION

Bird & Leng (1978*a*) showed that in cattle fed on low-protein–molasses-based diets, considerable increases in productivity could be achieved by removing rumen protozoa, and Martin *et al.* (1968) demonstrated a need to feed a restricted amount of high-quality forage to cattle fed on molasses-based diets to establish an efficient rumen function and therefore animal production.

The major object of the study reported here was to obtain information on the dynamics of the small species of protozoa, which appear to predominate in the protozoal population in cattle fed on *ad lib.* diets of molasses (Bird & Leng, 1978*a*) or starch (Schwartz & Gilchrist, 1975), in order to gain more understanding of their role in the nutrition of the ruminant. The experiment was organized so that protozoal dynamics were studied using two diets, one high in fibre and low in molasses, the other low in fibre and high in molasses. These diets were chosen in order to establish different populations of protozoa. The diets were expected to produce vastly different fluid kinetics as the low-fibre content of molasses-based diets has been shown to reduce outflow to very low rates (see Rowe *et al.* 1979; Beveridge & Leng, 1981). Although rumen volume was approximately 1·5 times greater in the steers fed on the low-forage diet compared with the heifers fed on the high-forage diet, actual outflow rate of fluid from the rumen was not different between diets. It is not possible to distinguish whether the larger rumen volume was due to the sex of the animal or the amount of forage, but it seems more likely to be the latter.

In cattle given sugar cane as a basal diet the predominant protozoa are holotrichs (Valdez *et al.* 1977); in the studies reported here the predominant protozoa were the small oligotrichs (mostly *Entodinium* spp.). In cattle fed on the high-fibre diet, the composition of the protozoal population was diverse with a reduction of the small oligotrichs and an increase in number of both the large oligotrichs (*Polyplastron* and *Epidinium* spp.) and the large ciliated holotrichs (*Isotricha* spp.). Although total numbers decreased, the larger mass of these protozoa maintained the biomass of protozoa in the rumen at a constant level (see Table 2) (the 'large' protozoa are from 60 to 100 times the biomass of the small protozoa as represented by *Entodinium caudatum* (see Clarke *et al.* 1982)). As these observations were made in two groups of cattle at different times, the factors that altered the composition of protozoal species in the rumen cannot be ascertained. However, as the dilution rate of fluid was highest on the high-forage diet (see Table 1), it is possible that fluid kinetics may affect protozoal species which are present in the rumen and this hypothesis is currently being researched.

Previous studies with sheep demonstrated that ^{14}C-labelled protozoa, prepared in the same way to that used here, were viable when injected into the rumen. To check whether this was also true for protozoa obtained from cattle fed on the diets used in the present study, a number of preliminary experiments were carried out to reaffirm that the marker is not subject to dilution in labelled protozoa as a consequence of their metabolism and cell turnover. The amount of ^{14}C relative to N in isolated protozoa incubated in vitro did not significantly change over 1400 min. Similarly, there was no change over a prolonged incubation period in the ^{14}C relative to N in labelled protozoa obtained from the rumen 26 h after the animal had received an intrarumen injection of ^{14}C-labelled protozoa. Over the same period, the specific radioactivity (μCi/g N) of the protozoal pool in the rumen declined according to first-order processes. This indicates that, for unknown reasons, the protozoa ceased to grow once they were removed from the rumen but kept in rumen fluid. At the same time, undoubtedly, protozoa in the pool sampled from the rumen continued to grow. However, ^{14}C-labelled methane was generated in vitro, indicating that even though protozoa were not growing, they were apparently releasing N and ^{14}C at the same rates. These results are in general agreement with previously reported observations (Leng, 1982) and suggest that protozoa, although not growing, were apparently lysing in vitro. A possible explanation for the failure of protozoa to grow in vitro is that they rapidly exhaust essential nutrients which are either continuously supplied in the rumen from the feed or from endogenous inputs, or are liberated when the protozoa are in close association with the fibrous component of digesta. The limiting nutrient in this case might be choline, which is very rapidly broken down by bacteria in the rumen (Broad & Dawson, 1976). Thus protozoa appear to be able to alter their growth rate from zero to levels that are determined by nutrient supply. This was also indicated in studies of large ciliates in the rumen of cattle fed on sugar-cane diets (Leng *et al.* 1981).

Estimates of VFA concentrations in rumen fluid were not available for one group of animals, and therefore no comparisons are possible between the two groups. However, for animals fed on the higher intake of forage, a relatively high proportion of propionate in the VFA was apparent. The percentage propionate in the rumen VFA of cattle fed on molasses is usually low but even between animals it is highly variable (see Beveridge & Leng, 1981).

When a diet is conducive to protozoal growth in the rumen, the population that develops is likely to be somewhat dependent on the rumen-fluid dynamics and rumen volume and, in the present study, more large protozoa appeared to favour conditions when fractional outflow rates of fluid were highest and rumen volumes were lowest. Dense populations of large protozoa in the rumen are present in cattle fed on ryegrass–clover pastures (Clarke, 1965) and they have a much slower growth rate and longer turnover time (Leng *et al.* 1986). However, the largest biomass of protozoa was found in the rumen of cattle fed on sugar cane where half-life varied from 5 to 15 d. There were no differences in rumen fluid outflow rates between animals fed on high- or low-forage levels in addition to molasses. The predominance of the small protozoa at the low fractional outflow rates of fluid may have little significance but it may be important to set up controlled experiments to examine the influence of turnover time of rumen liquid and the protozoal populations that develop.

The main conclusions from these studies are that the small ciliate protozoa in the rumen of cattle fed on molasses are apparently retained within the rumen to a lesser extent than the large ciliate species (e.g. *Isotricha*). Most of the irreversible loss from the protozoal pool in the rumen appears to be due to lysis of protozoa in the rumen rather than movement down the digestive tract. The present study, together with the other studies reported from these laboratories, suggests that because a large proportion of the protozoal pool turnover is within the rumen, protozoa are likely to decrease the efficiency of net microbial growth

since they utilize nutrients that are potentially available to the host animal and therefore increase the ratio, VFA:protein (amino acids) that are absorbed (see also Veira *et al.* 1984; Veira, 1986). It has been shown in a number of feeding situations where dietary protein is low, that supplementation with a protein that escapes rumen fermentation and is digested in the duodenum improves the efficiency of feed utilization with or without an increase in feed intake (see for review, Preston & Leng, 1987). This, together with the results presented here, helps to explain the increase in the efficiency of utilization of relatively low-protein diets by unfaunated sheep for production, particularly wool growth (Bird & Leng, 1985) and possibly milk and LWt gain.

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