Factors Affecting the Migration and Sequestration of Rumen Protozoa in the Family Isotrichidae

By BURK A. DEHORITY* AND PATRICIA A. TIRABASSO

Department of Animal Science, Ohio Agricultural Research and Development Center,
The Ohio State University, Wooster, Ohio, USA

(Received 5 July 1988; revised 27 October 1988; accepted 14 November 1988)

A chemotactic migration of Isotrichidae, primarily *Isotricha* and *Dasytricha*, into sheep rumen contents was observed at feeding time, followed by an abrupt decrease in relative numbers, presumably as a result of sequestration. An increase in the numbers of isotrichids was also observed before feeding, and was not a response to handling or sampling of the animals. When feeding was delayed for 4–5 h, isotrichid numbers increased markedly, falling again when feed was provided. The size of the decrease in numbers after feeding was directly related to feed intake. In general, chemotactic migration of the Isotrichidae was observed 12 h after feeding, while migration without feeding occurred after 22–24 h. In animals normally fed once daily, the relative number of isotrichids in the rumen contents of an unfed animal about 3 h after the regular feeding time is probably the best estimate of total isotrichid numbers. Two migrations of the same isotrichids appeared to occur in animals fed twice a day. It is proposed that feeding level controls the amount of storage polysaccharide in the protozoan cell, which in turn controls migration of Isotrichidae into the rumen. The isotrichids appeared to sequester by settling in the ventral rumen.

INTRODUCTION

A diurnal cycle for *Dasytricha* was first observed in sheep by Purser (1961), who noted a rapid increase in their relative numbers in rumen fluid between 20 and 24 h after feeding. Warner (1966) observed a similar cycle in sheep for both *Dasytricha* and *Isotricha*. This cycle for Isotrichidae differs markedly from that exhibited by the entodiniomorphs, whose numbers decrease at feeding and begin to rise about 8 h later (Purser & Moir, 1959; Warner, 1966). The initial decrease in relative numbers of entodiniomorphs is presumably caused by physical dilution factors such as feed and water intake and salivation. The subsequent increase in the population results from protozoal growth in response to nutrients.

Warner (1966) suggested that the Isotrichidae undergo rapid multiplication within a 4–8 h period around feeding, and then do not divide for about 18 h. However, the numbers of dividing cells observed during the time of rapid increases in Isotrichidae in sheep (Warner, 1966), cattle (Dehority & Mattos, 1978) and water-buffalo (Michalowski, 1977) do not substantiate this explanation. Clarke (1965) found that numbers of Isotrichidae in cattle did not begin to increase until feeding time, peaking at 2 h postfeeding and then decreasing rapidly. Additional studies in cattle (Abe *et al.*, 1981; Murphy *et al.*, 1985) substantiated the observations of Clarke (1965), i.e. numbers did not begin to increase until feeding. However, other studies in sheep (Dehority, 1970), water-buffalo (Michalowski, 1975) and goats (Abe *et al.*, 1981) all reported an increase in the numbers of all Isotrichidae prior to feeding.

Abe *et al.* (1981) observed a thick protozoal mass on the wall of the reticulum of steers slaughtered after overnight starvation. When viewed under the microscope, the mass was found to consist primarily of Isotrichidae protozoa. Based on volume estimates, the mass consisted of about 95% *Isotricha*. They proposed that the Isotrichidae sequester on the reticulum wall after

feeding and then migrate back into the rumen contents when the animal is fed. Possible mechanisms for migration of the Isotrichidae could be a response to a chemical stimulus (chemotaxis), quantity of feed, the act of ingesting feed, contraction of the reticulum at feeding or some combination of all of these.

Since Orpin & Letcher (1978) were able to show chemotaxis of *Isotricha* to sucrose, glucose and fructose, it appears highly probable that the Isotrichidae migrate in response to soluble sugars in the incoming feed. Infusion of 3 litres of distilled water, artificial saliva, 0.45 M-NaCl or a corn starch slurry into the reticulum of two steers at normal feeding time had little if any effect on numbers of Isotrichidae (Murphy *et al.*, 1985). However, a fivefold increase of Isotrichidae was noted in the rumen contents when 0.76 M-glucose was infused (this amount of glucose was calculated to equal the soluble carbohydrate content of the ration). The present study was undertaken to study those factors which might control the migration of Isotrichidae into the rumen contents, particularly the migration which seems to occur just before feeding.

METHODS

Animals and diet. Six rumen-fistulated sheep, 1–2 years old and ranging in weight from 37.7 to 45.5 kg, were used in these experiments. Several months before the start of this study, all sheep were inoculated one or more times with sheep rumen contents which contained fairly high numbers of Isotrichidae ($1-6 \times 10^4$ ml⁻¹). Unless stated otherwise, the animals were fed 800 g daily of a complete pelleted ration containing 45% corn cobs, 35% alfalfa meal, 13.1% oats, 5% molasses, 0.4% urea and 1.5% mineral–vitamin supplement. Composite samples of contents from various locations within the rumen were collected through the fistula with the aid of a rigid plastic tube (inside diameter 1.5 cm). When sampling time coincided with feeding time, rumen contents were removed and the animals were fed immediately.

In experiments where rumen contents were removed, a 'Shop-Vac' commercial wet vacuum cleaner was used to take out as much of the contents as possible. Some of the residual contents could then be removed by hand with the rigid plastic sampling tube. The volume of contents removed was replaced with an equal volume of warm anaerobic dilution solution (Bryant & Burkey, 1953).

Where reported, statistical significance was determined with the paired *t*-test. Subsampling, fixation and counting procedures have all been previously described (Dehority, 1984).

Terminology. Before 1980, rumen ciliates were classified in two subclasses, Holotricha and Spirotricha (Honigberg *et al.*, 1964). The genera *Buetschlia*, *Isotricha*, *Dasytricha* and *Charonina* were all included in the subclass Holotricha and commonly referred to in the literature as holotrichs. Based on taxonomic data obtained by electron microscopy, a revised classification of the protozoa was published in 1980 (Levine *et al.*, 1980). The subclass Holotricha became obsolete, with the genus *Buetschlia* now classified under the subclass Gymnostomatia and the three remaining genera under the subclass Vestibuliferia. *Charonina* is classified in the family Blepharocorythidae, while *Isotricha* and *Dasytricha* are both classified in the family Isotrichidae. The term isotrichid is used as an abbreviation in this study to denote protozoa in the last two genera.

RESULTS

Effects of handling, feeding time and sampling

For the first experiment, all six sheep were adapted to handling for 15 d. The normal routine consisted of the following steps: (a) enter pen at 0800 h with sampling tube, move sheep into sampling position and simulate removal of rumen fluid, leave barn; (b) return to barn at 0850 h, empty feed bunks and weigh-out feed; (c) feed sheep at 0900 h. Fig. 1 presents the results of a typical experiment with four sheep showing the variation observed in numbers of Isotrichidae around feeding time. For sheep 3 and 4, isotrichid numbers increased prior to feeding, while numbers increased after feeding in sheep 4 and 6. In a replicate experiment (data not shown) all of the sheep were observed to show increases before feeding, after feeding or both. Presumably this increase in number results from migration of the isotrichids into the rumen contents.

In the next set of experiments, feeding was delayed for 4.5 h. The sheep were all handled as described for the previous experiment. Fig. 2 presents the results from one of these experiments and shows that numbers of Isotrichidae began to increase in sheep 3, 4 and 6 just before the normal feeding time and continued to increase for 3–4.5 h. When feed was offered, numbers

Fig. 1. Changes in numbers of isotrichids in relation to feeding time. The sheep were adapted to a standard procedure for handling, sampling and feeding (see text). $\circ$, Sheep 3; $\blacktriangle$, sheep 4; $\square$, sheep 5; $\bullet$, sheep 6.

Fig. 2. Effect of delayed feeding on numbers of isotrichids. $\circ$, Sheep 3; $\blacktriangle$, sheep 4; $\square$, sheep 5; $\bullet$, sheep 6. Normal feeding time was 0900 h.

dropped immediately, with no apparent chemotaxis to incoming feed. It is assumed that this decrease in numbers is the result of sequestration of the isotrichids, either on the walls of the reticulum (Abe *et al.*, 1981) or some other as yet unknown site in the rumen. When the numbers of isotrichids did not continue to increase after feed was withheld, as in sheep 5, a chemotactic response to feeding was observed. This experiment was repeated in all four sheep on two additional days, with similar results. If the handler did not enter the barn until 3.5 h after the normal feeding time (1230 h) and fed at 4.5 h, similar cycles were observed, i.e. isotrichid numbers were high at 3.5 h and decreased rapidly after feeding. Thus the isotrichids apparently migrated into the rumen without any known outside stimulus.

Fig. 3 presents the results of a series of experiments with sheep 4, where the animal was first sampled 3, 2 or 1 h before feeding. Results of the previous studies were substantiated, in that personnel entering the barn and sampling the animal at various times before feeding did not appear to affect isotrichid numbers. Increases were noted before feeding, after feeding or both, and the increase before feeding occurred even when the animal was not sampled previously. A marked increase in isotrichid numbers occurred when sheep 4 was sampled and fed 3 h before the normal feeding time (Fig. 3). Similar data were obtained for these experiments using three other sheep.

Further studies were done with four of the sheep, on the possible influence of personnel entering the barn and handling the animals before feeding. Essentially there were three

Fig. 3. Effect of initial sampling time and early feeding on numbers of isotrichids in sheep 4. ○, Sampled 3, 2 and 1 h before feeding; ▲, sampled 2 and 1 h before feeding; □, sampled 1 h before feeding; ●, fed 3 h before normal feeding time. Normal feeding time was 0900 h.

Table 1. *Effect of prior sampling on numbers of isotrichids*

Experiment no.	Sheep no. . . . Sampling time (h) . . . Feeding time (h)	10 ⁻⁴ × No. of isotrichids ml ⁻¹							
		2		3		4		6	
		0700	0900	0700	0900	0700	0900	0700	0900
I	0900	3.02 ^a	6.18*	2.12	2.53	1.44 ^a	2.37*	1.02 ^a	2.11*
II	0700	5.23 ^b		2.28		2.48 ^b		2.91 ^b	
III	0900	3.38 ^{ab}	5.18*	1.52	1.98	2.14 ^b	1.98	1.76 ^{ab}	2.94*

* For each animal, means in the same row are significantly higher ($P < 0.05$) at 0900 h.

^{ab} Within a column, means without a common superscript differ at $P < 0.05$.

experiments, each repeated on five separate days (Table 1). The experiments were: (I) sample at 0700 and 0900 h, feed at 0900 h; (II) sample and feed at 0700 h; and (III) a repeat of experiment I. Adaptation periods were 7 d for I, 5 d for II and 3 d for III. When the animals were sampled at 0700 and 0900 h and fed at 0900 h, isotrichid numbers were significantly higher ($P < 0.05$) at 0900 h in three of the four animals in experiment I and two of the four animals in experiment III. Isotrichid numbers increased significantly ($P < 0.05$) at 0700 h in these animals between experiments I and II; however, the decreases at 0700 h between experiments II and III were not significant. These data would substantiate the observation that isotrichid numbers increase before feeding and although there is considerable between-day and between-animal variation, this increase occurs without any previous handling or stimulation of the animals.

Feeding level

The effect of feed level on numbers of Isotrichidae was investigated using four sheep, each fed a different level of feed one day a week, i.e. 50, 100, 150 and 200% of the amount of feed given on all other days (800 g). The data from all animals were combined to produce the graphs shown in Fig. 4. The sheep were fed the different amounts at 0900 h. Numbers of isotrichids appeared to be inversely related to feed level, i.e. more sequestration apparently occurred with higher feed intakes. Although the mean differences appeared to be large, only the differences at 3 h after feeding (1200 h) were significant. Isotrichid numbers at the 50% feed level were greater than all

Fig. 4. Influence of feed level on numbers of isotrichids. Normal feed level (100%) was 800 g. $\circ$, 50% of normal feed level; $\blacktriangle$, 100%; $\square$, 150%; $\bullet$, 200%. Values are means of results from four sheep.

Fig. 5. Effect of feed level on changes in numbers of isotrichids resulting from feeding 12 and 24 h after normal feeding time. Different amounts were fed at 0900 h on day 1, 50% of the normal daily ration was fed at 12 h and 100% (800 g) was fed at 24 h. Feed level and sheep no. were: $\circ$, 25% (sheep 2); $\blacktriangle$, 50% (sheep 3); $\square$, 100% (sheep 4); $\bullet$, 125% (sheep 6).

others at that time, and numbers at the 100 and 150% feed levels were greater than at 200% ($P < 0.05$). Variation both within and between animals was large. When the numbers of isotrichids at 1200, and at 0700 and 0900 h on day 2, were all combined, the total at the 50% feed level was significantly higher than all other levels ($P < 0.05$). The total at the 100% feed level was higher ($P < 0.05$) than at 150 and 200%, which were not different.

In a related experiment (data not shown), 20 g corn starch, 40 g sucrose and 20 g casein hydrolysate in 200 ml water were added to the rumen 30 min after feeding. Isotrichid numbers decreased to a greater extent with the addition of the soluble substrates.

To evaluate further the effect of feed level on the chemotactic response, the four sheep were each fed a different amount of diet, i.e. 25, 50, 100 or 125% of normal (800 g). After 12 h all animals were fed 50% of the normal amount and 12 h later were offered their normal ration. The results of this experiment (Fig. 5) indicate that the isotrichids in sheep at all feed levels showed chemotaxis to incoming feed at both 12 and 24 h. However, the magnitude of response appeared to be inversely related to initial feed level, i.e. larger increases with lower feed intake.

Fig. 6. Effect of feed level on changes in numbers of isotrichids resulting from feeding 4, 8 and 24 h after the normal feeding time. Different amounts were fed at 0900 h on day 1, 50% of the normal daily ration of 800 g was fed at 4 and 8 h and 25% of the daily ration was fed at 24 h. Feed level and sheep no. were: ○, 25% (sheep 2); ▲, 50% (sheep 3); □, 100% (sheep 4); ●, 125% (sheep 6).

Fig. 7. Effect of delayed feeding on changes in numbers of isotrichids in response to subsequent feedings. Sheep 2 and 4 were fed 25% of the 800 g daily ration at the normal feeding time of 0900 h on day 2. Sheep 3 and 6 were not fed. ○, Sheep 2; ▲, sheep 3; □, sheep 4; ●, sheep 6.

A somewhat similar experiment was done in which the four different amounts of feed were given at 0900 h, but 50% of the diet was then fed at 4 and 8 h. After 24 h, only 25% of the diet was fed to all animals. A small chemotactic response to feeding was observed at 4 h in the two animals initially fed the two lower feed levels (Fig. 6). No further response was elicited after an additional 4 h when most of the isotrichids appeared to sequester. A chemotactic response to feeding 25% of the diet was observed in all animals at 24 h. The increase in numbers was greater in those animals initially fed the lower amounts of feed. Some increase in the numbers of isotrichids was observed before feeding in the animal initially fed at the 25% level.

If feeding was delayed for 6 h, most of the isotrichids had apparently migrated into the rumen contents and little if any chemotaxis was observed (Fig. 7). A chemotactic response was noted 18 h later in sheep 2 and 4 when fed 25% of their normal diet. Sheep 3 and 6 were not fed 18 h later; however, the isotrichids began to migrate into the rumen contents, reaching a peak at 26 h after the previous feeding.

Fig. 8. Changes in numbers of isotrichids in relation to feeding 50% of the 800 g daily ration at 0800 and 1800 h. ○, Sheep 2; ▲, sheep 3; □, sheep 4; ●, sheep 6.

Frequency of feeding

At this point, it was unclear as to whether the isotrichids which migrated into the rumen contents in response to additional feeding at 12 h were the same organisms which had sequestered at the normal feeding time. Also, would these cells sequester and exhibit chemotaxis after an additional 12 h, at the next normal feeding time? In other words, did the isotrichids migrate on the basis of time or level of feed intake? Some of the present data suggest that feed level or intracellular concentration of storage polysaccharide might be the controlling factor. To investigate this question, the animals' feeding schedule was changed so that they received 50% of the normal 800 g diet at 0800 and 1800 h. Diurnal concentration curves for sheep 2, 3, 4 and 6 (Fig. 8) showed that the rate of sequestration of the isotrichids appeared to be somewhat slower after the 0800 h feeding than when all of the daily ration was fed at one time. The increase in numbers after feeding at 1800 h was markedly lower than after the morning feeding. Similar data were obtained in a replicate experiment.

Effect of removing the rumen contents

Fig. 9 shows the normal diurnal cycle for isotrichids in sheep 3 (day 1). On day 2, only 35% of the ration was fed at 0800 h and 800 ml of rumen contents were removed at 0830 h. After removal, an equal volume of warm buffer was added and the animal was fed 35% of its daily ration. The number of isotrichids in the rumen contents removed was $11.4 \times 10^4 \text{ ml}^{-1}$. The number of isotrichids which migrated into the rumen at 1700 h was markedly lower, and only this lower population of isotrichids was observed the next morning at 0830 h. These data suggest that the same isotrichids migrate into the rumen at both 0800 and 1700 h. Similar experiments were done with sheep 2 and 4 (data not shown) except the first day or normal diurnal cycle was not measured. The volume of rumen contents removed and the number of isotrichids were 1200 ml, $12.1 \times 10^4 \text{ ml}^{-1}$ and 1800 ml, $7.0 \times 10^4 \text{ ml}^{-1}$ for sheep 2 and 4, respectively. Results in both sheep were almost identical to the previous experiment.

For comparison, an experiment was done where rumen contents were removed 6 h after feeding, when the isotrichids were presumably sequestered (Fig. 10). Removal of 1900 ml of rumen contents at 1400 h had little effect on the concentration of isotrichids at 1700 h or 0830 h the next morning. The first 1800 ml of rumen contents removed contained 2.0×10^4 isotrichids ml^{-1} . The last 100 ml removed with the plastic tube contained 13.7×10^4 isotrichids ml^{-1} , suggesting some sequestration in the ventral part of the rumen. The total number of isotrichids removed in this experiment was 5.0×10^7 . This compares to 9.1×10^7 , 14.5×10^7 and 12.6×10^7

Fig. 9. Effect of removing rumen contents 30 min after feeding on subsequent numbers of isotrichids (sheep 3). The total daily ration was 800 g. On days 1 and 3, 50% of the daily ration was fed at 0800 and 1700 h. On day 2, 35% of the ration was fed at 0800 h; 800 ml of rumen contents were removed at 0830 h and replaced by the same volume of anaerobic buffer; 35% of the ration was then fed. ○, Day 1; ▲, day 2; □, day 3.

Fig. 10. Effect of removing rumen contents 6 h after feeding on subsequent numbers of isotrichids (sheep 3). The total daily ration was 800 g. On day 1, 1900 ml of rumen contents were removed at 1400 h and replaced by the same volume of anaerobic buffer. ○, Day 1; ▲, day 2.

Fig. 11. Effect of removing rumen contents 6 and 7 h after feeding on subsequent numbers of isotrichids (sheep 3). The total daily ration was 800 g. On day 1, 3425 ml of rumen contents were removed at 1400 h and replaced with anaerobic buffer. At 1500 h, 2650 ml rumen contents were removed and replaced with anaerobic buffer. ○, Day 1; ▲, day 2.

total isotrichids removed in the previous experiments, where isotrichid numbers were markedly reduced at 1700 h and 0830 h the next day.

Using sheep 3 again, the above experiment was repeated, except in addition to removal of the rumen contents and replacement with buffer at 6 h after feeding, contents were again removed 7 h after feeding (Fig. 11). It appears that many of the sequestered isotrichids were removed by this procedure. At 6 h the number of isotrichids was $1.7 \times 10^4 \text{ ml}^{-1}$ and at 7 h, $3.4 \times 10^4 \text{ ml}^{-1}$. The total number of isotrichids removed was 14.8×10^7 , which is comparable to the numbers in those experiments where rumen contents were removed 30 min after feeding. Thus, enough isotrichids were removed to affect markedly the numbers available to migrate in response to incoming nutrients at 1700 h or the next day at 0800 h.

DISCUSSION

The results obtained in this study clearly indicate that the number of isotrichids in rumen contents is variable and influenced by time of sampling, feed level and frequency of feeding. In an animal fed once daily, numbers can remain about the same or more than double in the 3 h before feeding (Figs 1 and 3). The chemotactic response to feeding varies with feed level as does the number of isotrichids after feeding (Figs 5, 6 and 8). In general, the maximum number of isotrichids, which occurs at or just after feeding, appears to be an equilibrium between migrating and sequestering isotrichids. If one samples rumen contents more than 2–3 h after feeding, most of the isotrichids will probably be sequestered (Figs 1, 3 and 4).

Data presented in Figs 2 and 7 suggest that in animals fed once daily, the number of isotrichids in the rumen contents of an unfed animal approximately 3 h after the normal feeding time would probably be the best estimate available of the total number of isotrichids. In most cases isotrichid numbers begin to increase just before or at the normal feeding time and continue to increase for 3–4 h. At that time, numbers begin to decrease whether the animal is fed or not. Presumably the decrease in isotrichid concentration reflects a lack of energy for maintenance of the protozoan cell (Potter & Dehority, 1973). Since there is apparently little or no increase in isotrichid numbers in response to incoming feed, it might then be assumed that few, if any, of the isotrichids are still sequestered.

The amount of polysaccharide stored in the isotrichid cell is probably a function of feeding level, and may control migration of the isotrichids into the rumen. Essentially, it is proposed that the isotrichids begin to utilize stored polysaccharide shortly after they sequester. Approximately 10–12 h later, depending on previous feed intake, the level of storage polysaccharide has decreased below the chemotactic threshold level, i.e. that point at which the isotrichids will migrate in response to soluble sugars. If the animal receives no nutrients at that time, storage polysaccharides are further utilized until they reach a specific minimum level about 20–22 h after feeding. At this level of storage polysaccharide, the isotrichids will begin to migrate into the rumen. This latter migration might be termed a depletion response as compared to the chemotactic response discussed above.

Abe *et al.* (1981), based on visual observations, proposed that the isotrichids sequestered on the wall of the reticulum. They observed a thick protozoal mass after overnight starvation, which could readily be dislodged or dispersed by vigorous agitation of the reticulum wall. We have observed in the present study and previously that when samples of rumen contents are removed from sheep about 30 min after feeding, and placed in a large beaker, white masses visible to the naked eye form on the top, bottom and at various layers in the contents. Microscopic observation of this material revealed that it is composed primarily of isotrichids, similar to the protozoal mass described by Abe *et al.* (1981).

Sequestration may occur as a result of the densely filled isotrichids settling to the bottom of the rumen, and not solely by attachment on the reticulum wall. The data shown in Fig. 10 indicate that a large number of isotrichids occur in the bottom of the rumen. In another experiment (Fig. 11), removal of contents, addition of buffer and removal of contents 1 h later markedly reduced

the number of isotrichids. This would be considered a rather mild treatment and since no soluble sugars were added, chemotaxis should not be a factor. Thus, in addition to possible sequestration on the wall of the reticulum the isotrichids appear to settle in the ventral rumen. If they adhere to the wall, the association seems to be quite loose and readily disrupted.

This research was supported by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Manuscript no. 98-88.

REFERENCES

- ABE, M., IRIKI, T., TOBE, N. & SHIBUI, H. (1981). Sequestration of holotrich protozoa in the reticulo-rumen of cattle. *Applied and Environmental Microbiology* **41**, 758-765.
- BRYANT, M. P. & BURKEY, L. A. (1953). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *Journal of Dairy Science* **36**, 205-217.
- CLARKE, R. T. J. (1965). Diurnal variation in the numbers of rumen ciliate protozoa in cattle. *New Zealand Journal of Agricultural Research* **8**, 1-9.
- DEHORITY, B. A. (1970). Occurrence of the ciliate protozoa *Butschlia parva* Schuberg in the rumen of the ovine. *Applied Microbiology* **19**, 179-181.
- DEHORITY, B. A. (1984). Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Applied and Environmental Microbiology* **48**, 182-185.
- DEHORITY, B. A. & MATTOS, W. R. S. (1978). Diurnal changes and effect of ration on concentrations of the rumen ciliate *Charon ventriculi*. *Applied and Environmental Microbiology* **36**, 953-958.
- HONIGBERG, B. M., BALAMUTH, W., BOVEE, E. C., CORLISS, J. O., GOJDICS, M., HALL, R. P., KUDO, R. R., LEVINE, N. D., LOEBLICH, A. R., JR, WEISER, J. & WENRICH, D. H. (1964). A revised classification of the phylum protozoa. *Journal of Protozoology* **11**, 7-20.
- LEVINE, N. D., CORLISS, J. O., COX, F. E. G., DEROUX, G., GRAIN, J., HONIGBERG, B. M., LEEDALE, G. F., LOEBLICH, A. R., III, LOM, J., LYNN, D., MERINFELD, E. G., PAGE, F. C., POLJANSKY, G., SPRAGUE, V., VAVRA, J. & WALLACE, F. G. (1980). A newly revised classification of the protozoa. *Journal of Protozoology* **27**, 37-58.
- MICHALOWSKI, T. (1975). Effect of different diets on the diurnal concentrations of ciliate protozoa in the rumen of water buffalo. *Journal of Agricultural Science* **85**, 145-150.
- MICHALOWSKI, T. (1977). Diurnal changes in concentration of rumen ciliates and in occurrence of dividing forms in water buffalo (*Bubalus bubalus*) fed once daily. *Applied and Environmental Microbiology* **33**, 802-804.
- MURPHY, M. R., DRONE, P. E., JR & WOODFORD, S. T. (1985). Factors stimulating migration of holotrich protozoa into the rumen. *Applied and Environmental Microbiology* **49**, 1329-1331.
- ORPIN, C. G. & LETCHER, A. J. (1978). Some factors controlling the attachment of the rumen holotrich protozoa *Isotricha intestinalis* and *I. prostoma* to plant particles *in vitro*. *Journal of General Microbiology* **106**, 33-40.
- POTTER, E. L. & DEHORITY, B. A. (1973). Effects of changes in feed level, starvation and level of feed after starvation upon the concentration of rumen protozoa in the ovine. *Applied Microbiology* **26**, 692-698.
- PURSER, D. B. (1961). A diurnal cycle for holotrich protozoa of the rumen. *Nature, London* **190**, 831-832.
- PURSER, D. B. & MOIR, R. J. (1959). Ruminal flora studies in the sheep. IX. The effect of pH on the ciliate population of the rumen *in vivo*. *Australian Journal of Agricultural Research* **10**, 555-564.
- WARNER, A. C. I. (1966). Diurnal changes in the concentrations of microorganisms in the rumens of sheep fed limited diets once daily. *Journal of General Microbiology* **45**, 213-235.