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Sequestration of Holotrich Protozoa in the Reticulo-Rumen of Cattle

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Studies were carried out to determine the means by which holotrich protozoa can maintain their numbers within the rumen against the washout effect associated with the flow of ingesta. When a diet composed of 2 kg of concentrate and 1.5 kg of rice straw was fed to Holstein cows, about a fourfold increase in holotrich numbers per ml of rumen fluid was observed within 1 h after the commencement of feeding, and an abrupt decrease followed. This fluctuation in numbers was not related to the time of feeding. A sole feeding of 2 kg of concentrate had almost the same effect on the holotrichs as a sole feeding of 1.5 kg of rice straw. Administration of either 2 kg of concentrate or 1.5 kg of rice straw through the rumen fistula caused similar changes, though the extent of response to the former was greater than that to the latter. The administration of either 0.7 kg of starch or 0.2 kg of glucose through the fistula had a relatively minor effect on the holotrich population. Addition of rice straw to 0.5 kg of concentrate increased the change in numbers, but its addition had little, if any, effect when 1 kg of concentrate was fed. These results suggested that the fluctuation in holotrich numbers was related not only to the nature or component of feed but also to other factors such as the quantity or volume of a diet and the act of ingesting feed. Increasing the number of feedings up to eight times per day at 3-h intervals caused a decrease in the peak heights of holotrich numbers per milliliter of rumen fluid. A thick protozoal mass which primarily consisted of holotrichs was found on the wall of the reticulum of Holstein steers slaughtered after overnight starvation. These findings suggest that holotrichs would usually sequester on the reticulum wall and migrate into the rumen only for a few hours after feeding, and that this mode of behavior would be essential for holotrichs to maintain their population within the rumen of cattle. Possible mechanisms of the migration are also discussed.

Large numbers of protozoa are usually maintained in the rumen, though their division rates measured in vitro are almost inadequate for maintenance (17). A microbial rumination pool was postulated by Hungate et al. (18), and Abe and Kumeno (4) suggested that maintenance of the protozoal population may largely be dependent upon their removal from the rumen being at a slower rate than the outflow of rumen fluid. This was confirmed by other workers (12, 16, 27), and sequestration of protozoa within the rumen was postulated to explain their slower removal rate relative to the fluid turnover rate (27). This implies that a substantial portion of protozoal protein will be retained within the rumen (14, 27) and raises some questions as to the contribution of rumen protozoa to protein nutrition of the host (7-9, 23).

To date, however, very limited evidence has been obtained to support the theory of sequestration. Warner (26) stated that sequestration would be of only minor significance for most of entodiniomorphs judging from their diurnal variations in numbers. But the recent studies by Bauchop and Clarke (6) have demonstrated a firm attachment of *Epidinium* to feed particles within the rumen. Clarke (11) predicted that the phenomenon may well apply to similar entodiniomorphs (e.g., *Ophryoscolex*). Sequestration of the rumen flagellate *Neocalimastix* close to the rumen wall was postulated by Warner (26), but was discounted by Orpin (19, 20).

Of the diurnal variations observed in the numbers of rumen protozoa, one of the most puzzling patterns is exhibited by holotrichs. They begin to increase in numbers at the time of feeding, or just before or after feeding, reach the maximum at feeding or, in many cases, within 1 or 2 h after feeding, and then decrease abruptly to the pre-feeding level (10, 13, 24-26). Initially, this fluctuation was attributed to sequestration of organisms among either feed particles or papillae on the rumen wall (24), but no experimental evidence has been obtained to support this hypoth-

esis. Clarke (10) attributed this fluctuation to a rapid multiplication and the successive bursting of cells due to overfilling with synthesized polysaccharide. However, normally very few dividing or bursting cells are seen at any time in the rumen, except on very rare occasions (13, 26). Recently, attachment of *Isotricha* to fresh plant particles during the first 2 h after feeding was reported (21, 22), but this seems not enough to explain the cause of the increase, even though it may be one cause of the decrease, in numbers of holotrichs in rumen fluid.

This paper investigates the cause of diurnal variations in the numbers of holotrichs within the rumen as well as the means by which these organisms can resist the washout effect due to the rapid turnover of rumen fluid.

MATERIALS AND METHODS

Feeding experiments. Holstein cows fitted with rumen fistulae and weighing about 650 kg were used. Usually, they received a maintenance ration composed of 4 kg of mixed concentrate and 3 kg of rice straw in two equal portions at 0830 and 1630. The mixed concentrate consisted of 37.2% cereal grain (corn and milo), 28.8% bran (wheat bran and defatted rice bran), 27.4% plant oil meal (soybean meal and rapeseed meal), 4% molasses, and 2.6% minerals and contained 18.5% crude protein, 6.5% crude fiber, and 48.8% nitrogen-free extracts. Rice straw was cut into about 5-cm lengths before use, and drinking water was available ad libitum.

On day 1, cow no. 1 was given as usual the daily ration on halves at 0830 and 1630, on day 2 she was given the diet in halves at 1030 and 1630, and on day 3 she was given the diet in halves at 1230 and 1630.

Four other cows were allotted to different treatments. Cows no. 2 and 3 were given 2 kg of concentrate at 0930 and 1.5 kg of rice straw at 1330 instead of the morning feeding. Cows no. 4 and 5 were given the diets in the reverse order. Cows no. 2 and 4 were given the diet orally, and for cows no. 3 and 5 the diet was administered directly into the rumen through the fistula.

After a period of more than 3 weeks when the usual maintenance ration was fed, these cows were allotted to new treatments. Two cows were administered 0.7 kg of corn starch or 0.2 kg of glucose through the fistula instead of the morning feeding. To one cow (no. 4) starch was administered at 0930, and glucose was given at 1330, whereas to another cow (no. 5), these were administered in the reverse order. At first corn starch was administered as powder into the rumen of cow no. 4, and the corn starch immediately formed large clumps inside the rumen, some of which remained until the time of administration of glucose. It was believed that this might exert some influence on the holotrich population, so starch was administered into the rumen of cow no. 5 at 1330 after the starch was suspended in a small quantity of warm water. Glucose was given in solution in each case.

The other two cows were fed 6 kg of concentrate and 3 kg of rice straw per day in two different manners. Cow no. 2 was given the concentrate in 12 portions of 0.5 kg each at 2-h intervals for 24 h starting at 0830. Cow no. 3 was given the same quantity in six portions of 1 kg each at 4-h intervals. Both cows received 1.5 kg each of rice straw at 0830 and 1630. After 3 weeks of feeding the maintenance ration, these two cows received 8 kg of concentrate and 6 kg of rice straw per day in eight even portions (1 kg of concentrate and 0.75 kg of rice straw per feeding) at 3-h intervals starting at 0830.

Observations inside the rumen and reticulum. Observations were taken with fattening Holstein steers in a slaughter house. Details of feeding conditions before slaughter were not clear, but in general these steers had been reared on a high concentrate diet with restricted amounts of rice straw, and were taken to the slaughter house before 1700 at the latest on the day before slaughter, and then killed between 0900 and 1100 hours on the next morning after overnight starvation. Immediately after slaughter, the reticulo-rumen was cut off from the esophagus and the lower gut, and the contents of the rumen and reticulum were carefully removed.

Counting of protozoa. Samples of rumen fluid, about 200 ml at each time interval, were taken through the fistula. The fluid was strained through two layers of gauze and used for the counting of protozoa. One volume of rumen fluid was added to two volumes of 10% formaldehyde solution containing 300 mg of methyl green per liter of solution. Duplicate subsamples were prepared and stored at room temperature until counting. The stored subsamples were further diluted approximately with 30% glycerol solution, and protozoa were counted in a cell (10 by 10 by 0.4 mm) (2) at a magnification of ×100. All counts are the means of duplicate subsamples.

RESULTS

Figure 1 shows the results of the delayed feeding experiment with one cow for 3 successive days. In response to every delay of the morning feeding, a typical pattern of the fluctuation was delayed for the entodiniomorphs and the holotrichs. The numbers of entodiniomorphs per milliliter of rumen fluid decreased after feeding, but, on the contrary, about a fourfold increase was observed in the numbers of holotrichs within 1 h after the commencement of feeding, and then they decreased rapidly to the prefeeding level. It was clear that these changes in numbers corresponded to feeding of both 2 kg of concentrate and 1.5 kg of rice straw.

When either 2 kg of concentrate or 1.5 kg of rice straw was fed singly, the numbers of holotrichs fluctuated correspondingly to either feeding (Fig. 2). Also, when 2 kg of concentrate or 1.5 kg of rice straw was administered singly into the rumen through the fistula, similar fluctuations were observed, although the extent of response to rice straw seemed to be less than that of concentrate (Fig. 3).

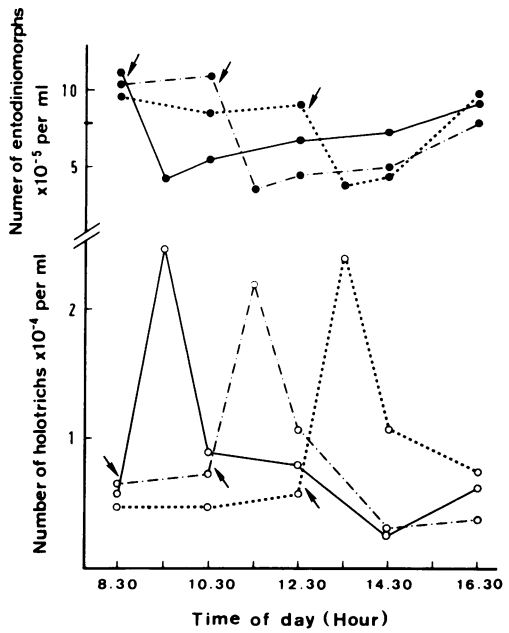

FIG. 1. *Changes in the concentration of holotrichs* (○) *and entodiniomorphs* (●) *within the rumen of one cow after feeding* (↓) *the diet consisting of 2 kg of concentrate and 1.5 kg of rice straw at 0830 on the day 1* (——), *at 1030 on day 2* (—·—·—), *and at 1230 on day 3* (·····).

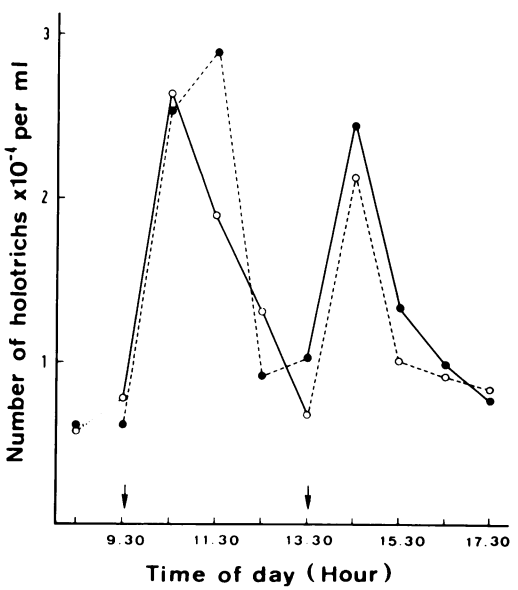

FIG. 2. *Changes in the concentration of holotrichs within the rumen of two cows, no. 2* (○) *and no. 4* (●), *after feeding of either 2 kg of concentration* (——) *or 1.5 kg of rice straw* (- - - - -), *alternately. The arrows denote the feeding time.*

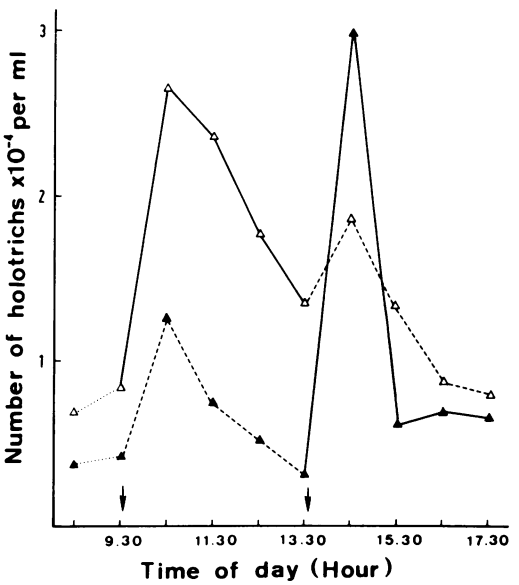

FIG. 3. *Changes in the concentration of holotrichs within the rumen of two cows, no. 3* (△) *and no. 5* (▲), *after administration of either 2 kg of concentrate* (——) *or 1.5 kg of rice straw* (- - - - -), *alternately, through the rumen fistula. The arrows denote the time of administration.*

When 6 kg of concentrate per day was fed in 12 portions of 0.5 kg each, the extent of fluctuation in the numbers of holotrichs after feeding was less than that observed when the daily amount was fed in six portions of 1 kg each (Fig. 4). The peak concentrations of holotrichs in rumen fluid were increased by the addition of 1.5 kg of rice straw to 0.5 kg of concentrate, but its addition had hardly any effect when 1 kg of concentrate was fed per feeding.

Administration of either 0.7 kg of corn starch or 0.2 kg of glucose into the rumen through the fistula caused some degree of fluctuation in the numbers of holotrichs per milliliter of rumen fluid (Fig. 5), but the extent was considerably less, at least in cow no. 5, than that observed when 2 kg of concentrate was given orally (Fig. 2) or through the fistula (Fig. 3). An apparent difference was seen between two cows in the fluctuation pattern after the administration of starch. This was regarded as having some relation to the difference in the administration method of starch through the fistula, suggesting that the formation of large clumps of starch exerted an influence on the population change of holotrichs in the rumen of cow no. 4.

When cows consumed a diet consisting of 1 kg of concentrate and 0.75 kg of rice straw in 3-h intervals for 24 h, the numbers of holotrichs per milliliter of rumen fluid fluctuated a good deal

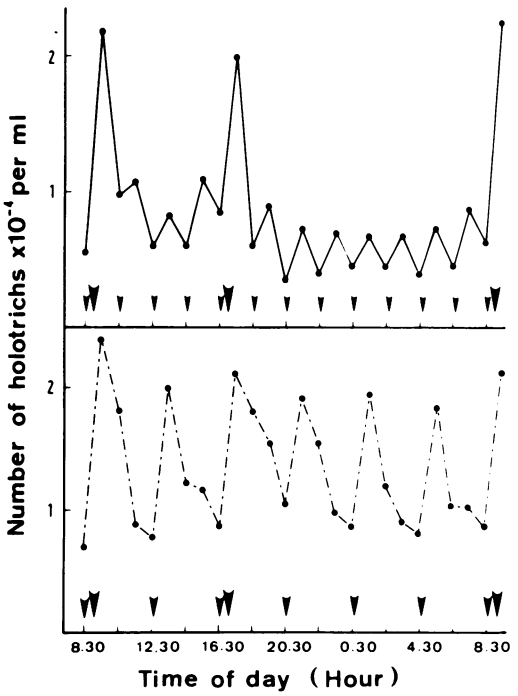

FIG. 4. *Fluctuations in the concentration of holotrichs within the rumen when 6 kg of concentrate per day was fed in 12 portions (——) or in 6 portions (— — — —). The smallest arrows denote the feeding time of 0.5 kg of concentrate, the middle arrows denote that of 1 kg of concentrate, and the biggest arrows denote that of 1.5 kg of rice straw.*

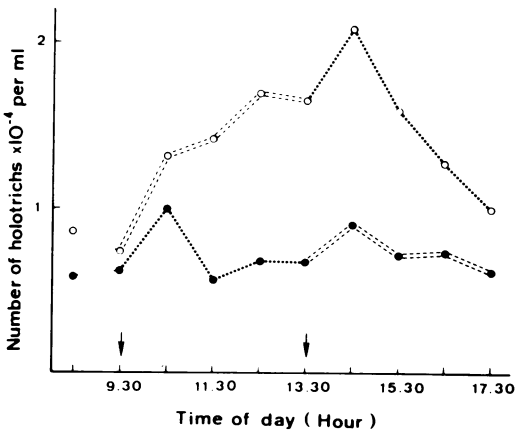

FIG. 5. *Changes in the concentration of holotrichs within the rumen of two cows, no. 4 (○) and no. 5 (●), after administration of either 0.7 kg of starch (===) or 0.2 kg of glucose (·····), alternately, through the rumen fistula. The arrows denote the time of administration.*

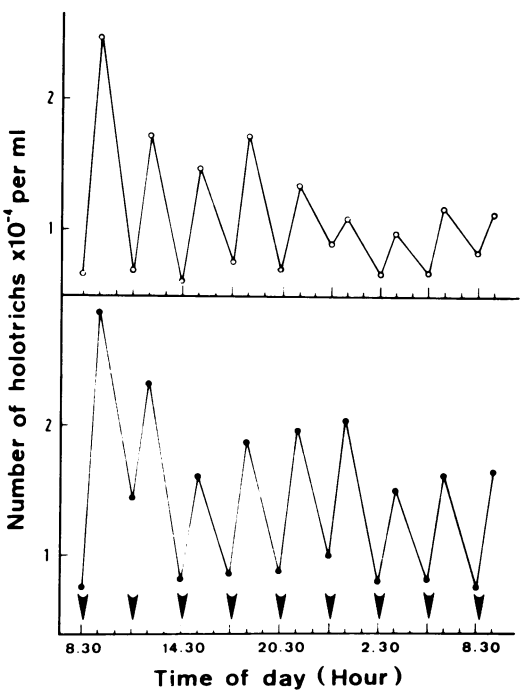

FIG. 6. *Fluctuations in the concentration of holotrichs within the rumen of two cows when a daily ration of 8 kg of concentrate and 6 kg of rice straw was given in eight even portions at 3-h intervals. The arrows denote the time of feeding 1 kg of concentrate and 0.75 kg of rice straw.*

in response to every feeding of the diet, but the peak concentration detected 1 h after every feeding showed a slow but steady decline (Fig. 6).

From observations inside the rumen and reticulum of steers slaughtered after overnight starvation, a thick protozoal mass that could be seen with the naked eye was found on the wall of the reticulum. The mass occurred among cells surrounded by folds in a shape of a beehive (Fig. 7A), and the mass was easily dissolved by a vigorous vibration of the reticulum wall (Fig. 7B). The protozoal mass was occasionally found when the reticulum was packed with feed particles, but was not found in other cases. Microscopic observation indicated that the mass was primarily composed of holotrichs, especially *Isotricha* (Fig. 8A and B). Counting the protozoa revealed that the mass consisted of 53% *Isotricha*, 25% *Dasytricha*, and 22% entodiniomorphs of the small type. When the proportion was computed in terms of volume by the method of Höller and Harmeyer (15), *Isotricha* made up about 95% of the mass, whereas entodiniomorphs did not exceed 1% (Fig. 9).

DISCUSSION

Holotrichs are usually a minority of the protozoal population in the rumen. In the aspect of

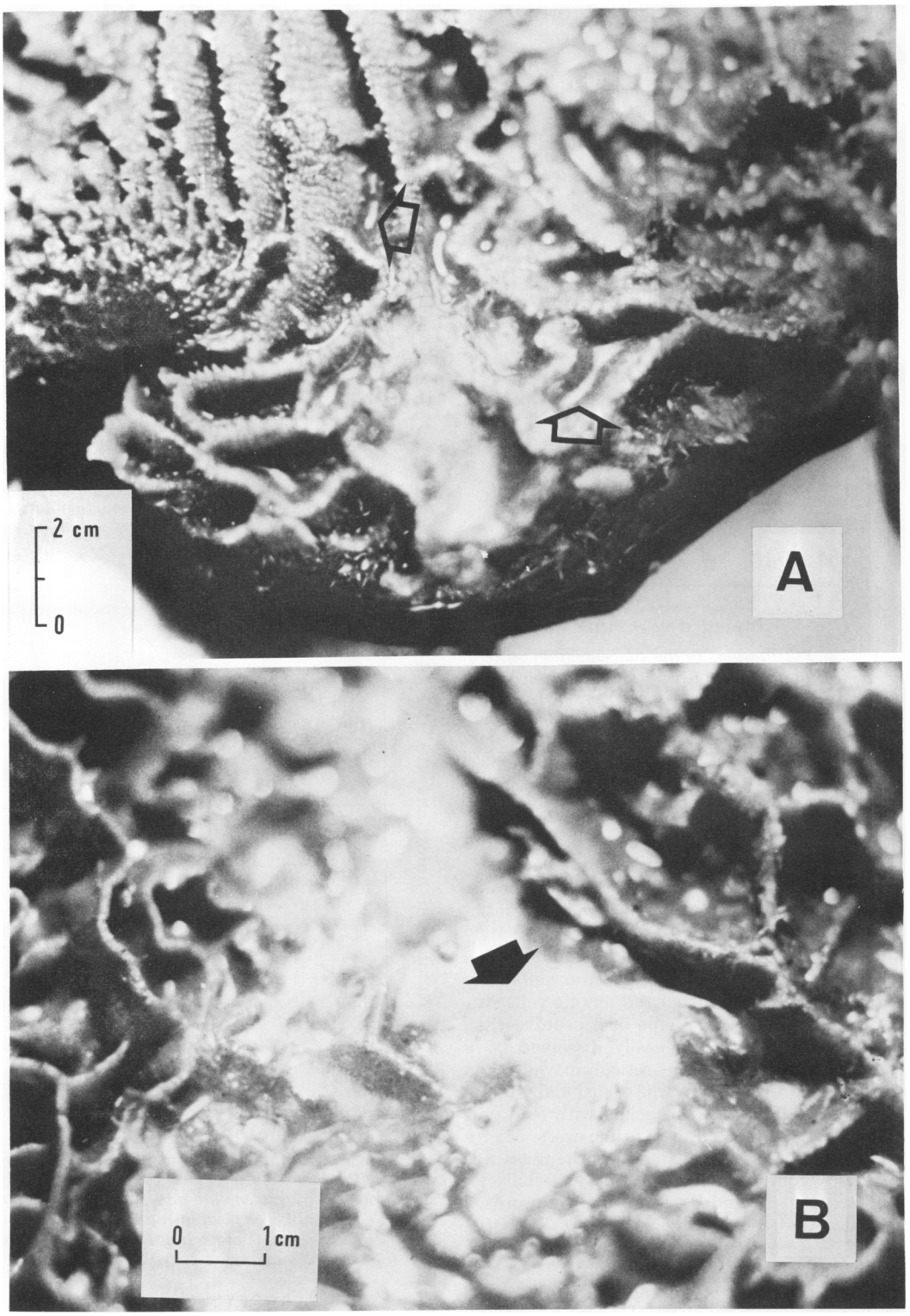

FIG. 7. *Protozoal mass (arrow) formed on the reticulum wall of steers slaughtered after overnight starvation: (A) a view immediately after the removal of the reticulum contents; (B) a view after vigorous vibration of the reticulum wall.*

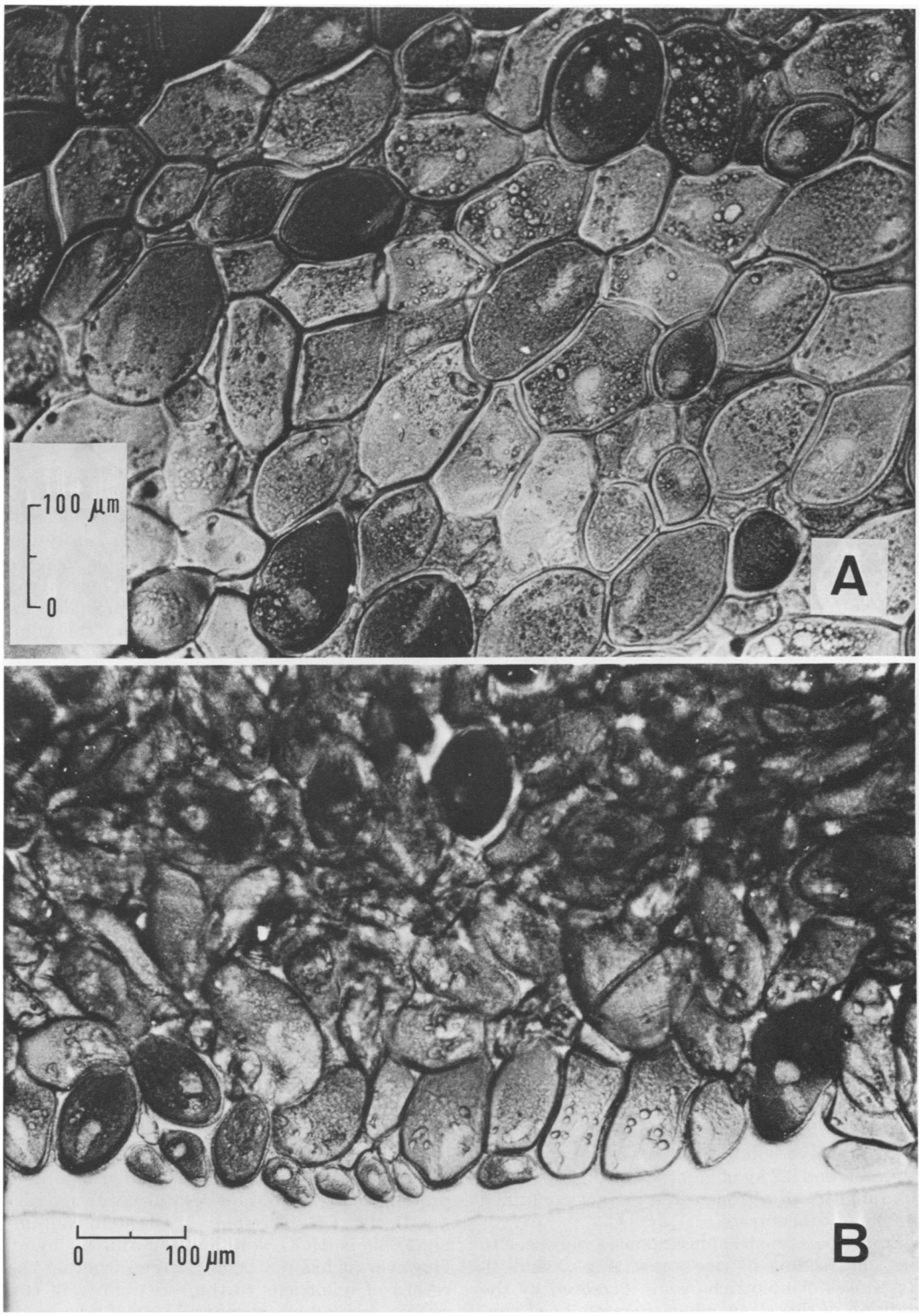

FIG. 8. *Microscopic views of the protozoal mass at a magnification of ×100. (A) A view immediately after a part of the mass was smeared on a slide. Holotrichs were stuck closely together and did not move even though the cilia were beating violently. (B) When a drop of isotonic salt solution was added to the smear, the mass dissolved rapidly, and Isotricha and Dasytricha began to move actively.*

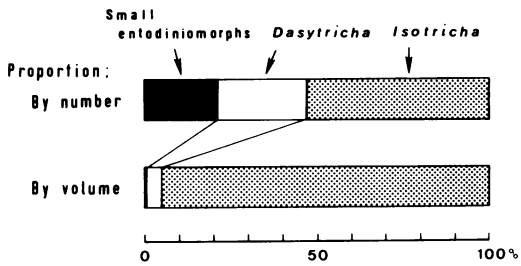

FIG. 9. *Constitution of the protozoal mass. Proportion by volume was calculated by the method of Höller and Harmeyer (15).*

volume or protein content, however, they are not always the minority, for Höller and Harmeyer (15) indicated that even when holotrichs are about 5% of the total population, they are responsible for over 35% by volume and for about 40% of the protozoal nitrogen. Therefore, it could be of significance for protein nutrition of the host animal whether the sequestration of holotrichs occurs within the rumen.

The results of the present studies seem to suggest that holotrichs would ordinarily sequester on the wall of the reticulum, with subsequent migration into the rumen for a few hours after feeding. One of the factors causing their migration would be a response to chemical stimuli originated from the diet (as shown in Fig. 1, 2, and 3). However, it seems inappropriate to consider the rapid migration immediately after feeding as simply the response to chemical stimuli from the diet. It has been shown that protozoal population is primarily affected by the type and amount of readily fermentable carbohydrates contained in a diet (1). Also, it has been generally accepted that holotrichs are active fermenters of soluble sugars, especially of glucose, fructose, and sucrose. In addition, *Isotricha* can assimilate grain starch, and *Dasytricha* can ferment maltose (17). However, the fluctuation in numbers of holotrichs was very limited when 0.7 kg of starch or 0.2 kg of glucose was administered into the rumen through the fistula (Fig. 5). Because the concentrate used in the experiments contained 48.8% nitrogen-free extract, and also assuming that the concentrate contained 35% starch and 10% soluble sugars, the sum of 0.7 kg of starch and 0.2 kg of glucose is almost equal to the quantity of nitrogen-free extract contained in 2 kg of concentrate.

Sometimes remarkable responses were seen to the sole feeding of rice straw (Fig. 2 and 3). Responses of holotrichs were increased by the addition of rice straw when the amount of concentrate was restricted (Fig. 4). It seems unlikely that rice straw contained many readily fermentable carbohydrates, since it has been previously shown that rice straw has little influence on the protozoal population, including holotrichs, in the rumen of cows (5). In addition, it seems that the procedure of administration of rice straw has some influence on the fluctuation of holotrich numbers (compare Fig. 2 and 3). These findings suggest that the quantity or volume of a diet as well as the act of ingesting feed may possibly be concerned with the fluctuation of holotrich numbers. Further, it brings to mind the possibility that the contraction of the reticulum at feeding may be involved in the fluctuation of holotrich numbers. This assumption might be supported by our observations that the holotrich mass was found when the reticulum was packed with feed particles, and that the mass was easily dissolved by vibration of the reticulum wall.

Some workers (23, 25) have reported a considerable increase in the numbers of holotrichs within the rumen of sheep even before the commencement of feeding. The results in the present work do not agree with these, but seem to agree with those of Clarke (10) and Dehority and Mattos (13), who observed no appreciable increase in holotrich numbers before the commencement of feeding in cattle. However, in preliminary experiments using goats, we have observed an appreciable increase in holotrich numbers before feeding. This prefeeding increase can hardly be explained by the multiplication theory, but it might be explained by the sequestration-migration theory proposed in this work as well as by the hypothesis that the contraction of the reticulum is involved in the migration. Presumably, in cows the contraction must occur in response to the actual behavior of ingesting feed, whereas in sheep and goats reflective contractions may occur in response to the stimuli surrounding the act of feeding. From experiments with goats we have obtained evidence which would support this speculation, and it will be reported in near future.

In addition, more detailed studies will be necessary to verify the hypothesis proposed in this work. Besides, the factors responsible for the holotrichs returning to the reticulum wall are somewhat unclear. In our in vitro studies (2), a protozoal mass similar to that observed in the present work was formed on the surface of a proteinaceous membrane (Naturin sausage casing; Becker Co., Germany) in a system containing 1.08% $NaHCO_3$ solution as perfusion liquid. However, it has not been clarified whether the return of holotrichs to the reticulum wall is a simple chemotactic response.

In summary, the results of the present work suggest that the cause of fluctuations in the

numbers of holotrichs per milliliter of rumen fluid can probably be attributed to their behavior of migrating rapidly from the reticulum into the rumen at feeding and sequestration again on the reticulum wall after a few hours. The results shown in Fig. 6 seem to indicate that the more frequently migration occurs, the more organisms are washed out from the rumen with fluid, suggesting that the behavior would be essential for these organisms to maintain their population within the rumen.

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