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# Adherent Bacterial Populations on the Bovine Rumen Wall: Distribution Patterns of Adherent Bacteria

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Fourteen tissue sites from the bovine reticulo-rumen were examined by scanning electron microscopy to determine the distribution patterns of bacterial populations adhering to the epithelium. Although diet variations did not appear to influence the total number of tissue-adherent bacteria present in adult Herefords, diet affected their distribution. It appeared that the distribution of the bacterial populations may be directly affected by the physical state of the digesta. The digesta may be mechanically removing adherent bacteria from the tissue surface by abrasive action. The total adherent population consisted of subpopulations with separate distribution patterns, and macropopulations of morphologically similar bacteria were occasionally observed at specific sites on the epithelial surface. Ureolytic organisms on the epithelium followed a distribution pattern considerably different from the general bacterial distribution.

Adherent bacterial populations found lining the gut wall have been studied in numerous animals (3, 5, 7, 12, 13, 19, 26, 30), and their distribution patterns along the digestive tract suggest an intimate interaction between the colonizing bacteria and their hosts. The bacteria often demonstrate preferential sites to which they will adhere, so that populations of a single species or morphological type can be found in specific locations in the digestive tract. This is true in human oral populations (20); in intestinal populations in pigs (17), mice (7, 12), and rats (11, 30); in the rumens of sheep (3) and cattle (24); and in the crops of birds (4, 18, 26).

In ruminants the bacteria in the rumen have access to relatively unmodified feed and may, therefore, exhibit greater dependence on diet (22), whereas the bacteria in the digestive tracts of monogastric animals may be as much influenced by the host's metabolism as by its diet (15, 16, 25).

It is likely that the tissue-adherent bacteria, because of their physical attachment to the epithelium, are more intimately associated with, and more closely tied to, the metabolic activity of the host than are the lumenal bacteria. The large number of ureolytic and facultatively anaerobic organisms associated with the rumen wall, as discussed in previous papers (9, 29), indicates that the adherent organisms are different from the lumenal populations as a result of their association with the epithelium. All the ureolytic activity associated with the epithelium is likely of bacterial origin (6, 14, 21, 27). Rumen wall-associated urease activity can be removed from the rumen with antibiotic treatment (8), and ureolytic activity of rumen tissue in newborn calves is negligible (9).

This present study was undertaken to observe the distribution patterns of the adherent bacterial populations throughout the reticulo-rumen of cattle and to determine whether the bacterial colonization patterns were affected by diet. Bauchop et al. (3) examined distribution patterns of bacteria in the sheep rumen, but no similar work has previously been performed on cattle.

## MATERIALS AND METHODS

**Animals.** Seven yearling Hereford bulls were used in this study. Four of the bulls received a self-fed ration of 60% barley, 10% oats, 10% beet pulp, and 20% chopped alfalfa hay (high-plane ration), whereas the other three bulls received chopped alfalfa hay (low-plane ration).

**Preparation of samples for scanning electron microscopy.** After sacrifice of the animals, tissue blocks (2 by 2 cm) were removed from 14 predefined reticulo-rumen locations (Fig. 1) in each animal and prepared for scanning electron microscopy by the thiocarbohydrazide method (24). These specimens were examined with a Cambridge Stereoscan 180 or a Hitachi 450 scanning electron microscope at an accelerating voltage of 20 kV.

**Analysis of populations.** A minimum of four random sites (each 1 by 1 mm) per tissue sample were examined by scanning electron microscopy. Each site consisted of a suitably oriented papilla or epithelial fold and was examined at a magnification of 1,000 to 2,000×. At these magnifications, large areas of tissue could be seen and bacteria could be distinguished from the epithelium.

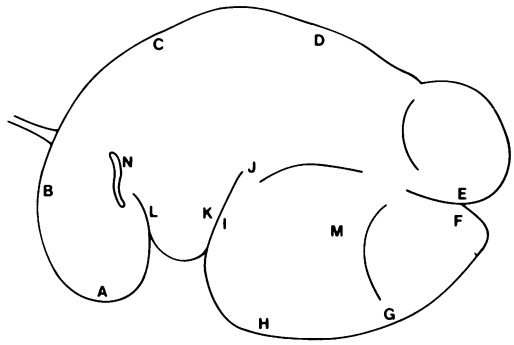


FIG. 1. *Diagrammatic representation of the reticulo-rumen from the left side indicating tissue sampling sites. The diagrammatic shape for the reticulo-rumen was taken from Balch (2). The sites sampled were as follows: A, ventral reticulum; B, cranial surface of the reticulum; C, dorsal surface of the cranial sac; D, dorsal surface of the dorsal sac; E, dorsal surface of the caudal pillar; F, ventral surface of the caudal pillar; G, ventral surface of the caudoventral blind sac; H, ventral surface of the ventral sac; I, caudal surface of the cranial pillar; J, lip of the cranial pillar; K, cranial surface of the cranial pillar; L, lip of the rumino-reticular fold; M, left wall of the caudoventral blind sac; and N, floor of the reticular groove.*

A scoring system was established by using a modified and extended version of that used by Bauchop et al. (3). The degree of bacterial colonization was scored from 0 to 4 based on arbitrarily defined standards presented below:

0, Consistently less than 10 bacteria on the tissue per field of view at a magnification of 1,500; 1, very low numbers of bacteria, usually only in microprotected regions; up to 25% of the tissue covered by bacteria; 2, moderate to low bacterial numbers; between 25 and 50% of the tissue covered by bacteria; 3, high numbers of bacteria present; between 50 and 75% of the tissue covered by bacteria; may be clumped or spread out evenly; 4, very heavy bacterial cover; consistent loading of the tissue by bacteria can be seen; greater than 75% of the the tissue densely covered by bacteria.

The above scoring and representative micrographs of each of the five population groupings were used together as references when the score for a site was determined.

When apparent trends in bacterial distribution were recognized, statistical analysis of the values was carried out to determine whether significant variations in the sampled populations existed between the animals or sites and to observe statistically derived trends.

The F test analysis of variance, or ANOVA (32), was used to determine whether significant differences existed ($P = 0.05$) between the sampled populations of each of the 14 subsites or between the cattle in the same feed regime.

Given that the differences in population distribution were significant, the Student-Neuman-Keuls test was applied to provide information on whether homogeneous subsets of the population could be defined (33). The calculations were made as part of a statistical package (SPSS) through Computer Services, The University of Calgary. An additional part of the program included the Bartlett-Box F test or the maximum variance/minimum variance test to test for homogeneity of variances (33). The application of these tests allowed for a detailed analysis of population trends that supplemented visual interpretation.

**Urease associated with the epithelium.** The ureolytic activity of tissue-adherent bacteria at various sites in the reticulo-rumen was determined by measuring the ureolytic activity of rumen tissue blocks. This was performed with the assumption that all ureolytic activity associated with the epithelium was of bacterial origin and that the tissue itself did not produce urease (6, 8, 14, 24, 27).

Urease was measured by our modification of the phenol hypochlorite method of Weatherburn (31) and Cook (10). Two adjacent tissue blocks (6.25 $cm^2$) were excised from each of 14 sites (Fig. 1), and within 10 min one tissue block was placed in 30 ml of reaction mixture (phosphate, ethylenediaminetetraacetic acid, urea) (10) and the other was placed in 30 ml of control mixture (no urea) and incubated for 1 h at 38°C. The reaction was stopped by pouring the reaction mixture into tubes, leaving the tissue behind. Duplicate 0.2-ml samples of the control and reaction mixture were mixed with 5 ml of reagent A, followed by addition and mixing of 5 ml of reagent B. Color was allowed to develop for 30 min or more and read at 625 nm on a Gilford 300 N spectrophotometer. The standard curve was prepared with ammonium sulfate. One unit of urease was defined as the amount of urease which catalyzes the hydrolysis of 0.5 μmol of urea to form 1 μmol of ammonia in 1 min at 38°C.

## RESULTS

Fourteen reticulo-rumen sites similar to those described for sheep by Bauchop et al. (3) were designated as sampling locations to provide a comprehensive overview of bacterial epithelial interactions occurring in that organ. A diagrammatic representation of the sampling sites is presented in Fig. 1.

Scanning electron microscopy of the sampling sites revealed that, in general, the bacteria were contagiously distributed over the epithelial cells. That is, the bacteria formed in clumps with the result that some microsites on the epithelial surface were completely covered by bacteria, whereas adjacent sites were not (Fig. 2). Regardless of the site sampled, highly exposed surfaces, such as tips of papillae or raised folds, tended to carry fewer adherent bacteria than the lower microprotected regions. Figures 3a and b, diagrammatic representations of the reticulo-rumen, facilitate interpretation of trends in the bacterial distribution by placing the distribution scores awarded at the approximate location from which they were derived. Out of a possible score

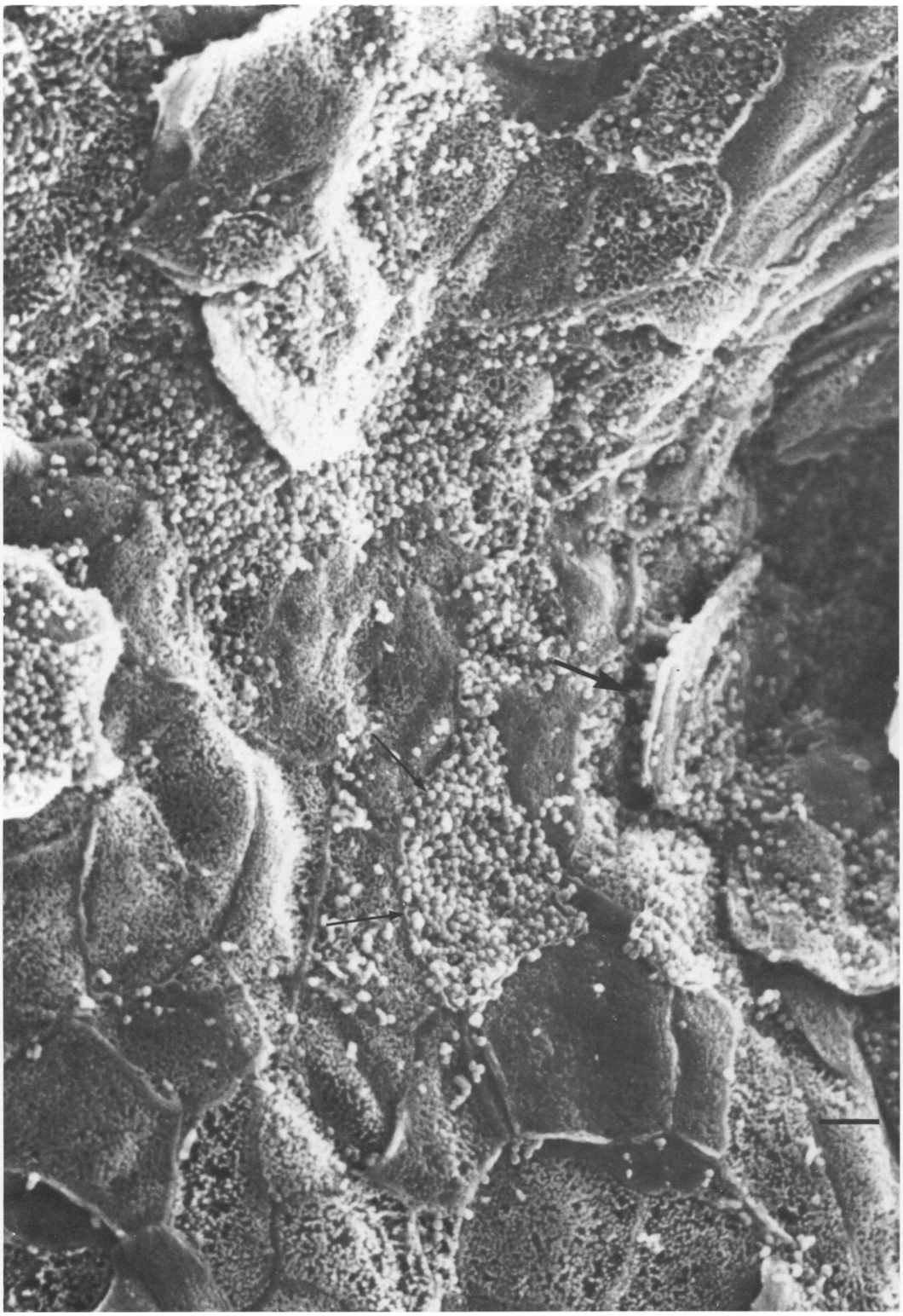

FIG. 2. *Scanning electron micrograph from a hay-fed calf, site D. The number of adherent bacteria vary from one epithelial cell to the next. One epithelial cell (small arrows) may be completely covered by adherent bacteria, whereas adjacent cells carry very low numbers. Bacteria can be seen colonizing the under surface of a sloughing epithelial cell (large arrow). The bar in this and the other scanning electron micrographs is 5 μm.*

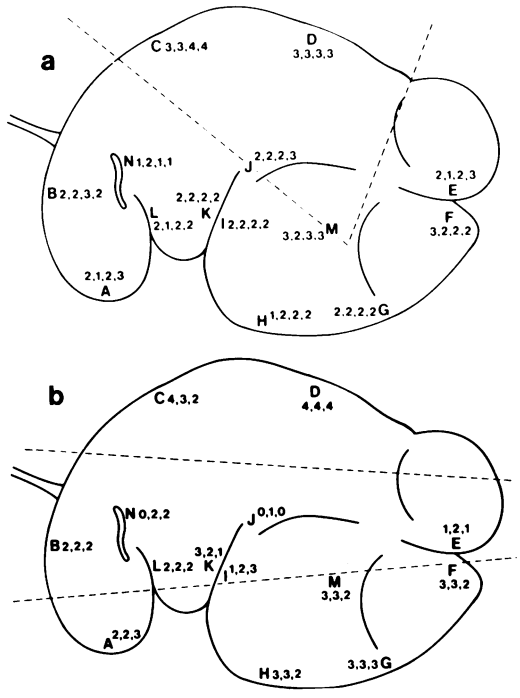


FIG. 3. *(a) Diagrammatic representation of the reticulo-rumen, indicating the distribution of the tissue adherent bacterial populations present in cattle fed a high-plane ration. Reading from left to right, the four scores presented at each location correspond to the four bulls fed a high-plane ration. The score can range from 0 to 4. The dotted line separates the two major bacterial subpopulations into high scoring sites (sites C, D, and M) and low scoring sites based on the results of the Student-Neuman-Keuls test. (b) Diagrammatic representation of the reticulo-rumen, indicating the distribution of the tissue adherent bacterial populations present in cattle fed low-plane diets. Reading from left to right, the three scores presented at each location correspond to the three bulls fed a low-plane diet. The scores can range from 0 to 4. The dotted lines separate the sites into high- and low-population subsets based on results of the Student-Neuman-Keuls test. The midline region, located between the two dotted lines, carried the low-density adherent populations, whereas the populations on the dorsal and ventral side of the midline carried the more dense populations. Site J, in the center of the midline region, formed a third subset as the site consistently carrying the lowest number of adherent bacteria.*

of 56 for each diet determined, the four high-plane and three low-plane ration animals scored an average of 31 and 31.3, respectively, suggesting that the total number of bacteria adhering to the rumen wall does not vary significantly with diet in adults.

Distribution of the population scores for animals receiving the high-plane ration indicated that the dorsal regions of the rumen maintained the largest adherent population, whereas the ventral regions were less well populated.

F test analysis of variance (ANOVA) indicated no significant differences in bacterial distribution between the four cattle on the high-plane diet, but demonstrated significant differences ($P = 0.05$) in the distribution of the populations throughout the 14 sites in each animal. The results of the Student-Neuman-Keuls analysis demonstrated a separation of the populations into three subsets ($P = 0.05$). The reticular groove (site N) with the lowest population score was alone as one subgroup and sites C, D, and M, with the highest scores, formed a second. All the other sites fell into the third grouping. A line separating the two major populations has been drawn through Fig. 3a.

Adherent bacteria in animals receiving the low-plane ration (Fig. 3b) demonstrated distribution patterns similar to those observed in high-plane ration animals. The dense bacterial cover, however, extended to the ventral regions of the reticulo-rumen. The F test ANOVA of low-plane ration animals indicated no significant difference in population distribution between the three animals tested, but indicated that significant differences existed between the bacterial population numbers distributed over the 14 sites examined ($P = 0.05$). Three homogeneous subsets were demonstrated by the Student-Neuman-Keuls test. The low-scoring lip of the cranial pillar (J) existed alone as one group, and sites A, C, D, F, G, H, and M formed the high-population group which constituted the high dorsal and low ventral areas of the rumen. The positions of the high- and low-population groups are presented diagrammatically in Fig. 3b. The position in the rumen of the coarse fibrous component of the rumen digesta corresponds closely to this medial region (2).

In animals fed a low-plane ration, subpopulations consisting of bacteria of a single morphological type were observed. The dorsal sac (D) was consistently colonized by a lawn of cocci (Fig. 4) in each of the three animals studied, while sites B and F were populated by curved rods (Fig. 5). The lip of the rumino-reticular fold carried a high proportion of rods in one animal and a high proportion of cocci in another. Similar macrocolonies were not noted in any of the locations examined in the four animals fed high-plane rations.

The distribution of ureolytic bacterial populations on the epithelium of a low-plane ration animal was determined by direct measurement of ureolytic activity of unwashed tissue blocks (6.25 $cm^2$) derived from 13 reticulo-rumen sites. The results are presented diagrammatically in

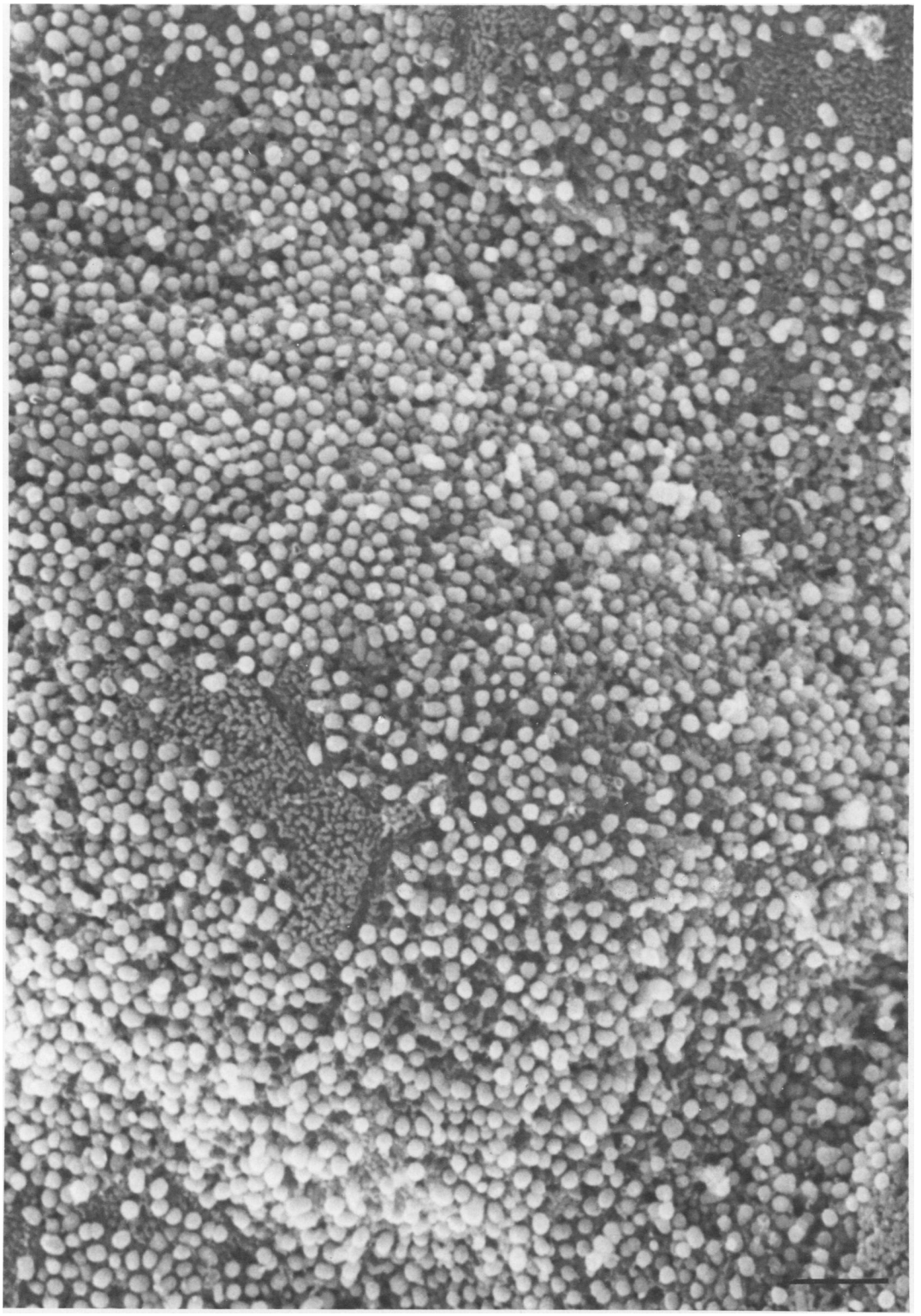

FIG. 4. *Scanning electron micrograph from a low-plane diet animal, site D. Large areas of the dorsal sac were covered by a lawn of cocci, as typified by the tissue-adherent bacteria in this figure.*

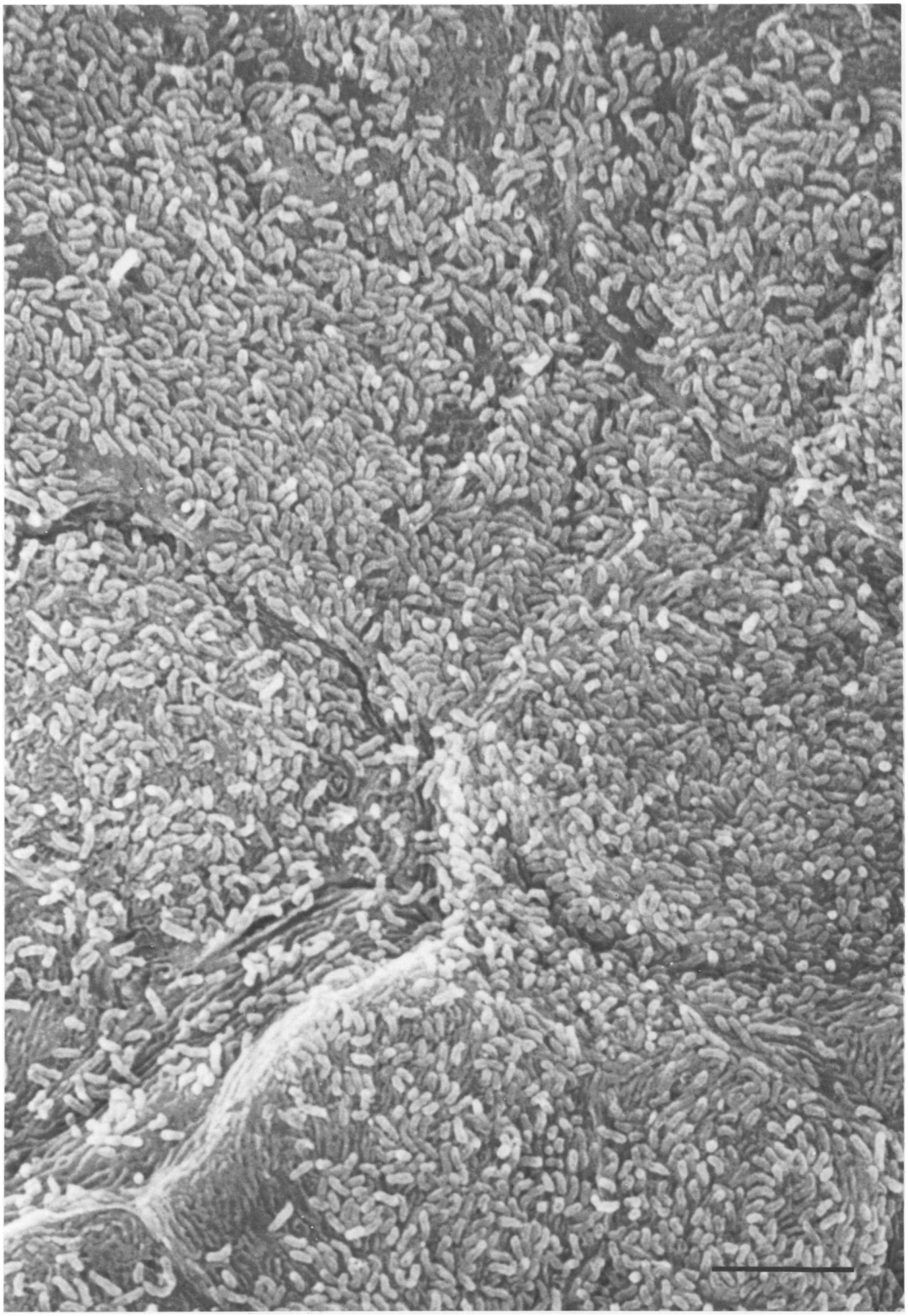

FIG. 5. *Scanning electron micrograph from a low-plane diet animal, site B. The population consists of a morphologically homogeneous bacterial population, consisting of curved rods adhering to the epithelium that are part of a macrocolony of curved rods that were observed at this and other sites.*

Fig. 6 to show the activity patterns throughout the reticulo-rumen. The tissue-associated activity was higher in the ventral region than in the dorsal region of the reticulo-rumen and did not follow the same distribution pattern as that seen for the overall population as determined by scanning electron microscopy. Many of the sites normally demonstrating a high population density recorded the lowest ureolytic activity. The midline area, excluding the lip of the cranial pillar (site J), recorded the highest urease activity, and yet it was the region with the lowest population levels in the three low-plane ration animals. These results cannot be conclusive, because they are derived from observations of a single animal, and this trend needs further examination.

## DISCUSSION

The distribution of the adherent bacteria on the rumen wall appears to be strongly influenced by the type of diet fed. Although the total number of adherent bacteria present on the epithelium did not change significantly between the two diet groups, there were noticeable differences in population distribution patterns.

Reticulo-rumen contractions in animals fed a low-plane ration will lead to distinct stratification of the digesta (2), and also apparently to stratification of the bacterial populations. A large gas bubble forms in the dorsal sac over a mat of rough fibrous material lying along a horizontal plane through the midline of the animal. Beneath the fiber layer and throughout the ventral region is a fluid layer consisting of fine, concentrated digesta. The location of the low bacterial population in the low-plane diet animal (Fig. 3b) closely corresponds to the position of the fibrous material in the rumen. Thus, the abrasive character of this stratified layer may be responsible for the low numbers present. The cranial pillar located in the middle of the fibrous zone with no papillae covering it and presenting a highly exposed surface yielded results which support this assumption. It carried the lowest population score of the 14 reticulo-rumen sites examined. The highest populations were at locations corresponding to the dorsal gas bubble and the ventral fluid region. These locations are seldom subject to the abrasive action of the plant fibers. Bauchop et al. (3) did not see the same trends when they studied the population distribution of adherent bacteria in the rumen of sheep fed a diet of white clover and perennial ryegrass. They found that the caudo-dorsal regions of the reticulo-rumen carried higher numbers of bacteria, whereas the cranial and ventral regions carried low numbers. They did not observe the horizontal separation of the rumen into a low midline population with higher bacterial populations located dorsally and ventrally to the midline. Also, site E in the sheep was consistently populated by high numbers of adherent bacteria, whereas in the cattle studied here site E consistently carried low population levels. These differences suggest that the distribution patterns of the adherent bacterial populations are not the same in sheep and cattle, and these differences may be due to species differences or to diet.

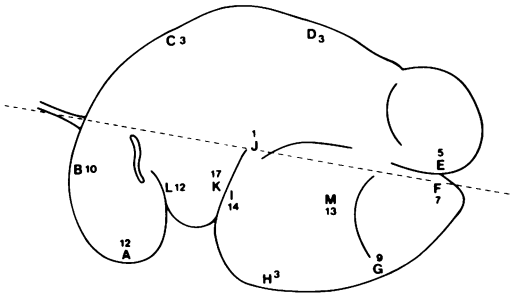


FIG. 6. *Diagrammatic representation of the reticulo-rumen of a low-plane diet animal, indicating the urease activities of 13 unwashed epithelial sites at the locations from which they were taken. A dotted line separates the dorsal sites, manifesting low activities, from the ventral sites, demonstrating high activities.*

In animals fed a high-plane ration, the consistency of the digesta changes very little throughout the rumen fluid (1). The bacterial distribution patterns in animals fed a high-plane diet (Fig. 3a) reflect this. The dorsal sac, where the air pocket is located, carried the highest number of adherent bacteria (similar to the populations in animals fed the low-plane ration), but most of the ventral sites were similar in their distribution scores, suggesting that all sites may have been abraded by the digesta to an equivalent extent. Comparisons between colonization scores at specific sites in animals fed low- or high-plane rations again suggest that feed character may influence distribution. Sites H and G in high-plane ration animals received scores similar to the rest of the ventral sac, whereas in the low-plane ration animals the fluid layer sites received higher distribution scores. In both feed groups, there were more bacteria in microprotected regions than in highly exposed areas, which again suggests that surfaces exposed to abrasive action carried lower bacterial populations.

The mechanical effects of digesta could influence bacterial numbers in two ways. The feed could physically remove bacteria from epithelial surfaces or could increase the rate of epithelial

cell desquamation and thus indirectly increase the rate of removal of the adherent bacteria. The uneven distribution of the bacteria on individual epithelial cells (Fig. 2) may reflect this situation. The number of bacteria present on each squamous cell may indicate the length of time that each epithelial cell has been exposed and available for colonization. Alternatively, results from research in other animal systems indicate that bacteria may exhibit different adhesion specificities for epithelial cells taken from the same location (23), and the clumped colonization patterns seen here may be an indication of this specificity.

The feed ration may affect the distribution of adherent bacteria at various locations in the rumen. The number of sites colonized by morphologically homogeneous macrocolonies were high in animals fed a low-plane ration which had the most highly structured or stratified rumen contents. The consistent occurrence of these macrocolonies at specific locations suggests that major selective factors have given these populations a colonizing advantage at these positions. This may involve an adhesion specificity between the bacteria and the epithelial surface, with stimulation of the specific adherent bacteria by some products or metabolites available at that site.

Assuming that epithelial ureolytic activity is due to tissue-adherent ureolytic bacteria, rumen wall colonization by ureolytic bacteria in the one animal examined did not follow the same distribution pattern as that of the total bacterial population in low-plane ration animals (Fig. 3b and 6). In the dorsal sac the highest recorded populations and the lowest rumen wall-associated urease activities were located. The ventral sites F, G, and H with high population scores manifested only moderate ureolytic activity. This suggests that microhabitats exist in the rumen and that selective factors which may differ from those affecting the overall population may be selecting for specialized adherent communities with distributions different from the overall populations.

Although the overall distribution of the colonizing bacteria may be governed by the physical state of the digesta, the ureolytic populations may be influenced by endogenous factors. The entry of urea across the rumen wall from the blood to the rumen represents the major endogenous route for nitrogen entry into the rumen (6). Since the proportion of blood flowing to the ventral rumen is double that flowing to the dorsal rumen (28), it is likely that along with the greater blood volume there would be an equivalent increase in the diffusion of blood components such as urea at these sites. This could explain why ureolytic activity was predominantly associated with the ventral regions.

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