RUMINAL FLORA STUDIES IN THE SHEEP

VI. DIURNAL, DAILY, AND SEASONAL FLUCTUATIONS IN THE CONCENTRATION OF "FREE" RUMEN BACTERIA AND IN RUMEN pH

By M. C. NOTTLE*

[*Manuscript received April* 9, 1956]

Summary

Diurnal, daily, and seasonal fluctuations in the concentrations of bacteria within the rumen and in rumen pH were determined in mature sheep.

Significant diurnal fluctuations were found in both bacterial concentrations and rumen pH, and marked differences occurred in the nature of the diurnal curve for bacteria in sheep which were fed two diets identical except for the protein source in the concentrate portion of the ration.

For each time of sampling (0, 3, and 6 hr after feeding) significant daily differences in rumen bacterial concentrations were observed.

In the seasonal variability trial, in which the sheep were fed a fairly constant diet for a period of nearly 2 years, a seasonal fluctuation in rumen bacterial concentration was found. The pH of the rumen was also found to fluctuate seasonally in a manner similar to the seasonal fluctuations in rumen bacterial concentrations. The minimum bacterial concentration and the minimum pH, which occurred in May, were significantly lower than the maxima which occurred in October.

The significance of these findings is discussed, and attention drawn to the similarity of the curves of hours of daylight and mean monthly temperatures and the seasonal curve of rumen bacterial concentration. The suggestion is made that light and temperature, as well as diet, may influence the seasonal fluctuation in rumen bacteria.

I. INTRODUCTION

The outstanding importance of the vast number of microorganisms in the rumen to the nutritional physiology of the sheep makes it imperative that the factors influencing the numbers and types of these organisms be thoroughly understood. True dietary effects can only be assessed if the fluctuations in rumen microbial population which occur under constant dietary conditions are known. To throw some light on this problem a series of experiments was undertaken with sheep fed single diets in which the diurnal, daily, and seasonal changes in the concentration of rumen bacteria were observed. Parallel observations on the changes in rumen pH were also made.

Several groups of workers have attempted to show that diurnal fluctuations in the concentration of rumen bacteria occur in sheep and cattle. Not only were inconsistent results obtained but different methods were employed. Thus Johnson *et al.* (1944) used plate counts, which were not specified as being anaerobic, and Bortree *et al.* (1946) and Chance *et al.* (1953) used the iodine staining reaction. The limitations of the plate counts are obvious, whilst the method used by the other workers, since the iodine staining reaction is dependent on the nutritional status of

* Institute of Agriculture, University of Western Australia, Nedlands, W.A.; present address: Department of Agriculture, Perth.

the bacteria, may or may not indicate fluctuations in concentration of the whole population. Henneberg (1922) has shown that the rumen population can be divided into iodophilic and non-iodophilic bacteria. Moir and Williams (1950) made direct counts of the "free" bacteria in rumen liquor after staining with aniline blue, and found that the concentrations obtained by this means agreed well with those obtained by the nigrosine smear method of Gall, Stark, and Loosli (1947). This latter method, rather than more specific methods, was chosen for the present study as it seemed to offer a better assessment of the ability of different diets to support a bacterial population.

Marked seasonal fluctuations in rumen bacterial concentration were observed in grazing sheep by van der Wath and Myburgh (1941) and Moir (1951). These fluctuations were attributed to seasonal changes in the nutritional quality of the herbage grazed. The possibility of such seasonal fluctuations occurring in sheep or cattle consuming diets of relatively constant composition has not previously been investigated, so far as is known.

II. Experimental

The investigations undertaken, together with diets, methods of feeding, numbers of animals in each determination, sampling times, and the observations made at each sampling time are presented in Table 1.

(*a*) *Design*

In each experiment a randomized-block design was used and the significance of all results, except feed and water consumption in the diurnal and daily experiments, was tested by means of the analysis of variance.

(*b*) *Animals*

Mature Merino wethers of similar age, weight, and appearance were selected at random from a large group and allocated to each of the experiments, except that, as far as possible, the same sheep were used in the second series of experiments on diurnal variations as were used for these experiments in the previous year.

(*c*) *Diets*

The daily diet used in the diurnal variation experiments consisted of 550 g oaten chaff plus 183 g of a concentrate mixture having the following composition: cracked wheat 82·5 g, wheaten starch 55·0 g, casein or wheat gluten 45·1 g, molasses 30 g, calcium carbonate 3·65 g and sodium chloride 3·65 g. In the first experiment the diet contained gluten and in the second casein, so that the two diets were identical except for the main protein source.

All weighings of the dietary components were made on the air dry material with the exception of the molasses which was weighed as the liquid. The concentrate materials were mixed in a concrete mixer, sprayed with a solution of molasses, and the mixture dried in a current of air at 55°C. The chaff and concentrate portions of the daily diet were weighed into a single bag and fed together in the first experiment

(1950) and into separate bags and fed simultaneously in separate feed boxes in the second experiment, as indicated in Table 1. The percentage crude protein in the gluten and casein diets were 9·4 and 10·4 per cent. respectively.

TABLE 1
EXPERIMENTAL DETAILS

Variability Tested	No. of Sheep	Diet	Method of Feeding Diet	Sampling Times	Observations
Diurnal	6	Chaff and gluten concentrate	Fed together	0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hr after feeding	Rumen bacterial concentrations, feed, and water consumption
Diurnal	6	Chaff and casein concentrate	Fed separately	0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hr after feeding	Rumen bacterial concentrations and pH, feed, and water consumption
Daily	5	Chaff and casein concentrate	Fed separately	0, 3, and 6 hr after feeding on each of 6 consecutive days	Rumen bacterial concentration, feed, and water consumption
Seasonal	4	Chaff and casein concentrate	Fed together	0, 3, and 6 hr after feeding on each of 2 successive days on eight occasions at approximately 3-monthly intervals over a period of 652 days	Rumen bacterial concentration and pH, feed, and water consumption

In the daily variability trial the casein diet was fed as just described. In the prolonged seasonal variability experiment the casein diet described above was used throughout, but the chaff and concentrate portions were fed together except during the rumen sampling of period 1, when each portion was fed in a separate feed box. Owing to storage limitations, mixing of concentrate at approximately 3-monthly intervals was necessary. This, together with unavoidable changes in the source of the chaff, resulted in some variability in the chemical composition of the rations as fed (Table 2). The crude fibre content was remarkably constant throughout but the protein content varied appreciably. The significance of this variation in composition is assessed below.

A progressive decline in feed consumption which was observed prior to period 3 suggested the possibility of cobalt deficiency. Analyses revealed that the diet was extremely low in cobalt and contained suboptimal amounts of copper. Both elements were incorporated in a drench and given for the first time on the 235th day of the experiment, i.e. 11 days after the completion of period 3. This was followed by a reduction in feed consumption which did not occur with later drenches containing cobalt only. Subsequently, adequate copper and cobalt were added to the diets.

(*d*) *Feeding Regime*

In each experiment the animals were fed daily at 9 a.m. At each rumen sampling period any feed residues were weighed and returned to the feed boxes after each sample had been taken. Adequate supplies of tap water were available to all sheep at all times, and in the seasonal variation experiment each sheep was drenched once a week with an oil rich in vitamins A and D except when this was within 7 days of rumen sampling.

TABLE 2

COMPOSITION OF THE RATIONS AS FED IN SEASONAL VARIABILITY TRIAL

Period	Dry Matter (g)	Crude Fibre (g)	Nitrogen (g)	Crude Protein in Dry Matter (%)
1	673	139	11·2	10·4
2	651	142	11·5	11·0
3	664	144	12·6	11·9
4	657	144	11·5	10·9
5	666	137	9·2	8·7
6	662	144	10·0	9·4
7	662	144	9·5	9·0
8	667	147	8·3	7·8

(*e*) *Sampling and Treatment of Rumen Material*

Rumen samples were obtained by stomach tube and pump (Moir and Williams 1950). An aliquot was formalized quantitatively for bacteriological study, and, where pH was determined, it was measured by means of a glass electrode immediately after the withdrawal of the sample. The nigrosine smear method of counting, as described by Moir (1951) and discussed by Williams and Moir (1951) was used. This enables counts to be made of all the "free" bacteria in the sample greater than 0·5 μ in diameter.

To determine diurnal variability, rumen samples were taken from each of six sheep in two trials at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hr after feeding (Table 1). Daily variability was assessed by taking rumen samples at 0, 3, and 6 hr after feeding from five sheep on each of 6 consecutive days. In the seasonal variability trial, rumen samples were taken from each of four sheep on 2 consecutive days, at 0, 3, and 6 hr after feeding, on eight occasions spaced at approximately 3-monthly

intervals throughout the 655 days experimental period. The first sampling coincided with the last day of the eight 10-day "collection" periods when digestibility and nitrogen balance determinations were made on each sheep.

III. Results

(a) *Diurnal Fluctuations*

The mean results for rumen bacteria and pH are presented in Figure 1.

(i) *Rumen Bacteria.*—For both the gluten and casein diets there were significant period (time after feeding) differences ($P<0{\cdot}001$) in the concentration of free rumen bacteria. Animal differences were also highly significant for the gluten diet ($P<0{\cdot}001$) and significant at the $P<0{\cdot}05$ level for the casein diet.

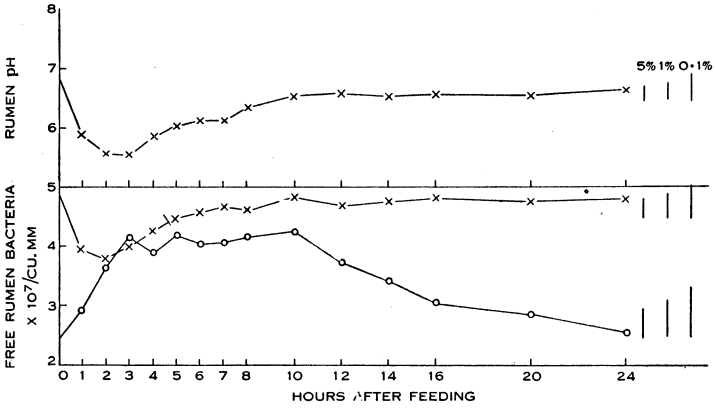

Fig. 1.—Diurnal fluctuations in free rumen bacterial concentration and rumen pH. ○ Gluten diet. × Casein diet. Least significant differences at the 5 per cent., 1 per cent., and 0·1 per cent. levels are indicated on the figure.

The diurnal variation curves were completely different for the two diets. With the gluten diet, the mean minimum bacterial concentration of 24 millions/cu.mm occurred immediately before feeding and rose to a maximum of 41 millions/cu.mm by 3 hr after feeding. The concentration remained at this level till 10 hr and thereafter slowly declined to pre-feeding level by 24 hr. The increase between 0 and 3 hr was highly significant ($P<0{\cdot}001$) but the 3–10 hr differences were not significant.

With the casein diet there was a sudden and highly significant ($P<0{\cdot}001$) decline from a maximum of 49 millions/cu.mm immediately prior to feeding to a minimum of 38 millions/cu.mm at 2 hr after feeding. Thereafter the concentration increased to pre-feeding level which was attained at about 10 hr. The differences between periods after 6 hr were, however, not significant.

(ii) *Rumen pH.*—No rumen pH determinations were made on the sheep receiving the gluten diet. On the casein diet period differences were highly significant ($P<0{\cdot}001$) but animal differences were not significant. It is apparent from Figure 1 that the diurnal trend in rumen pH was very similar to that of the rumen bacteria concentrations. Thus the maximum, pH 6·87, occurred at 0 hr followed by a signifi-

cantly lower ($P<0{\cdot}001$) minimum of 5·53 at 3 hr. Thereafter the pH slowly increased but the differences between periods after 10 hr were not significant. All periods were significantly less than at 0 hr ($P<0{\cdot}05$).

(iii) *Feed and Water Consumption.*—Wide variability was observed between animals in the rate of both feed and water consumption but consumption was generally faster and more uniform in the casein than in the gluten experiment. This is illustrated by the following average figures: an average of 361 g feed and 308 ml water were consumed in the first hour after feeding on the gluten diet, whereas on the casein diet an average of 569 g feed and 292 ml water were consumed in this period. By 3 hr after feeding the corresponding figures were 435 g and 441 ml, 617 g and 470 ml, respectively.

(*b*) *Daily Fluctuations*

The results for bacteria are presented in Figures 2 and 3.

(i) *Rumen Bacteria.*—Analysis of these data revealed that the following relationships were significant—days ($P<0{\cdot}05$), sheep ($P<0{\cdot}001$), time (after feeding) ($P<0{\cdot}001$), sheep×day interactions ($P<0{\cdot}001$), and day×time interactions ($P<0{\cdot}05$).

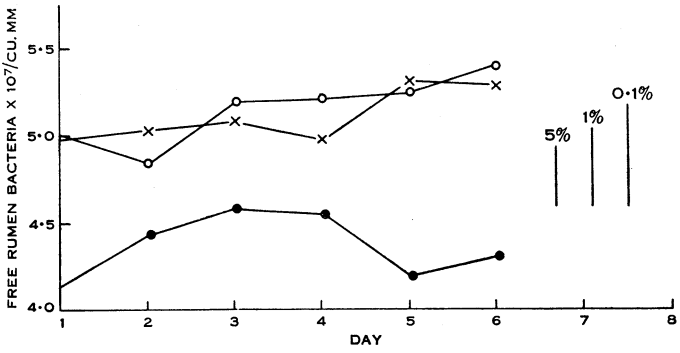

Fig. 2.—Daily variation in rumen bacterial concentration at three sampling times (time×day interaction). Each point is the mean for five sheep. ○ 0 hr, ● 3 hr, × 6 hr after feeding. Least significant differences at the 5 per cent., 1 per cent., and 0·1 per cent. levels are indicated on the figure.

It is apparent from Figure 2 that for each time of sampling (0, 3, and 6 hr after feeding) there are significant differences between days, but the days when these differences occur may differ between sampling times. It is evident also that the differences between sampling times are in agreement with those obtained with the casein diet in the diurnal variation experiment reported above.

The sheep×day interactions shown in Figure 3 indicate that while some sheep varied significantly in their daily means, others were relatively constant over the 6 days. The significance of the difference in means for individual sheep on any day also varied. Thus on the second day these differences were highly significant ($P<0{\cdot}001$) while on the fifth day they were not significant.

(ii) *Feed and Water Consumption.*—The average consumption of feed in the first 3 hr after feeding was 645 g, a total very close to that which occurred with this diet in the diurnal variation study. Water consumption, however, was much higher, averaging 1119 ml for the 3 hr.

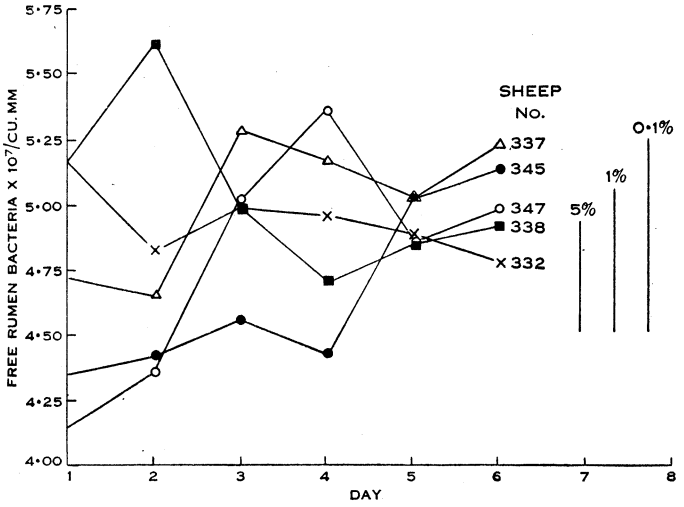

Fig. 3.—Daily variation in the rumen bacterial concentration for individual sheep (day × sheep interaction). Each point is the mean of three sampling times. Least significant differences at the 5 per cent., 1 per cent., and 0·1 per cent. levels are indicated on the figure.

(c) *Seasonal Fluctuations*

The mean results for all determinations, together with the least significant differences between any two periods, are given in Table 3.

In order to achieve statistical balance, the results for one sheep, which died from enterotoxaemia shortly after the fifth metabolism period, and the 3-hr values for rumen bacteria and rumen pH were omitted from the analyses. This was necessary because the 3-hr values were not available for periods 2 and 3. The results for rumen bacteria and pH therefore represent the mean of four sheep sampled on 2 consecutive days at 0 and 6 hr after feeding, while those for feed and water consumption are the mean amounts consumed over 6 hr on 2 consecutive days in each period. Sampling at these times, rather than at 0 hr only, was carried out in order to obtain some information on the nature of the diurnal pattern at different times of the year.

(i) *Feed and Water Consumption.*—With the exception of period 3, all sheep consumed the whole ration, so that the dry matter and nitrogen intakes are as given in Table 2. During period 3 a marked reduction in appetite and rate of food consumption occurred in three of the five animals. With the addition of cobalt both the rate and the total amount of food consumed increased so that the whole of the ration was consumed in subsequent periods. Differences in rate and amount of feed consumption between periods (other than period 3) were not significant. Differences between sheep

TABLE 3

MEAN EXPERIMENTAL RESULTS FOR THE SEASONAL VARIABILITY EXPERIMENT

Period number	1	2	3	4	5	6	7	8	Least Difference between Periods for Significance at		
Date	18.vi.51	24.ix.51	6.xii.51	12.ii.52	20.v.52	15.x.52	15.xii.52	9.ii.53			
Days on diet at commencement of determination	52	150	223	291	389	537	598	654	$P<0{\cdot}05$	$P<0{\cdot}01$	$P<0{\cdot}001$
Rumen bacteria (millions/cu.mm)	51·0	59·6	62·4	53·0	46·2	64·2	63·2	51·6	3·8	5·2	7·1
Rumen pH	—	6·9	6·6	6·7	6·3	6·8	6·5	6·6	0·15	0·21	0·29
Feed consumed in 6 hr (g)	724	643	279	733	683	733	665	729	169	230	310
Water consumed in 6 hr (ml)	1330	1150	863	1875	1050	1330	1236	1609	328	447	603

were also not significant, suggesting that the eating habits of the sheep were very similar.

Period differences in the rate of water consumption were significant ($P<0{\cdot}001$) but differences between animals and days were not significant.

(ii) *Rumen Bacteria.*—The concentrations of rumen bacteria differed significantly between periods ($P<0{\cdot}001$) and between times after feeding ($P<0{\cdot}001$). There were, however, no significant differences between days within periods, or between sheep, and the interactions period $\times$ time after feeding and days $\times$ time were not significant. The diurnal trend was similar to that reported earlier for the same ration.

Inspection of Table 3 indicates that there is a seasonal fluctuation in bacterial concentration. Covariance analysis showed that these highly significant period differences were not due to variations in the percentage protein in the diet. The following regression equation, which has no significant deviations, accounts for practically all period differences:

$$Y = 55{\cdot}0034 + 8{\cdot}7087 \sin(0{\cdot}9863(x-212))^\circ,$$

where Y is bacterial concentration in millions/cu.mm and x is the number of days after January 1st. The equation indicates that the bacterial concentration fluctuates sinusoidally about a mean of 55·0 millions/cu.mm and that the minimum and maximum concentrations occur on the 121st and the 303rd day of the year, i.e. on May 1st and October 30th. At these times they are 46·3 and 63·7 millions/cu.mm, respectively. This seasonal trend is similar to that reported for grazing sheep by van der Wath and Myburgh (1941) and by Moir (1951), but the seasonal fluctuations are much smaller, due in all probability to the much more constant composition of the diet in the present experiments.

Qualitative examination of the bacterial population in each period was not made, but it was evident from the nigrosine smears that the appearance of the population in period 3 differed from that of all other periods. The most striking feature of this period was the appearance of large forms, and of *Oscillospira* species in one animal, whereas in previous and subsequent periods the population consisted mainly of small forms. If cobalt deficiency was responsible for these changes, the effects contrast greatly with those of Gall *et al.* (1949) who observed a considerable reduction in bacterial concentration in cobalt deficiency but only slight qualitative changes.

(iii) *Rumen pH.*—The results presented in Table 3 indicate that the pH of the rumen fluctuates seasonally, in a manner similar to the seasonal fluctuations in rumen bacterial numbers. The minimum, which occurred in May, is significantly lower than the maximum which occurred in September and October. In addition to these significant period differences, sheep and days within period differences were also significant ($P<0{\cdot}05$).

The diurnal trend throughout the experiment was the same as that reported earlier in the diurnal experiment.

IV. Discussion

The very different diurnal curves of rumen bacterial concentration which occurred in the sheep on the gluten and the casein diets, in which only one component

of the whole diet was varied, indicates that comparisons between bacterial concentrations of animals on different diets should only be made when the nature of the diurnal curve on each diet is known. The finding that the greatest changes in bacterial concentration occur immediately after feeding indicates further that rumen samples should be taken as distant as possible from the immediate influence of feeding. With one daily feeding this, of course, means at 24 hr.

The greater percentage protein in the casein diet combined with its greater physical if not chemical availability, would be expected to favour a greater bacterial proliferation. No such effect is evident. On the other hand, the dilution of rumen contents by feed and water would presumably be larger on casein than gluten. The absence of evidence for any dilution on gluten suggests that other factors are involved in determining the diurnal curve. One possibility is the difference in the physical properties of the two concentrates. Whereas gluten enmeshed all other components of the concentrate, and the "nuts", when immersed in water, became swollen, glutenous, and retained their shape, the components of the casein concentrate were only slightly bound together by casein and the nuts disintegrated completely in water.

What these changes in concentration mean in terms of the total number of bacteria in the rumen at any time is unknown, but it is apparent that a method of determining rumen volume is required so as to take into account variations in volume which occur through feed and water consumption, saliva inflow, the passage of digesta from the rumen, and the passage of water into or out of the rumen through the rumen wall.

The diurnal fluctuations in rumen pH confirm the findings of several groups of workers (Monroe and Perkins 1939; Phillipson 1942; Myburgh and Quin 1943; and Chance *et al.* 1953) that there is a fall in rumen pH after the ingestion of food. The rate at which this fall occurs has been shown to be related to the availability of the substrate to the rumen bacteria (Phillipson and McAnally 1942). This factor, combined with the rapid consumption of the concentrates, probably accounts for the greater rate and extent of the pH changes in these experiments than in those of the authors cited above.

The small but significant day to day variations in rumen bacterial concentrations found in sheep receiving the same diet fed in the same way conflict somewhat with the findings of Moir and Williams (1950) and Williams and Moir (1951). These workers obtained excellent agreement between days when the same sheep were sampled at the same time on each day. The random nature of the small daily variations in the present experiment suggests that they were largely a reflection of daily differences in the manner and rate of the animal's eating and drinking.

The seasonal fluctuations in rumen bacterial concentration which occurred under relatively constant dietary conditions are of great interest, especially as very similar seasonal trends were obtained by van der Wath and Myburgh (1941) and Moir (1951) where the sheep were exposed to marked seasonal differences in the quantity and quality of the herbage grazed. The dietary conditions in the present experiments, although highly constant compared with those in the earlier work just cited, were subject to some variations, mainly in crude protein content, due to the use of different

batches of chaff and concentrate. The seasonal fluctuations in rumen bacteria which occurred could not be related to any such changes in composition of the ration. It seems therefore that some factor or factors other than diet were responsible for the seasonal changes in rumen bacterial concentration.

In Figure 4 the curves of hours of daylight (sunrise to sunset) and mean monthly temperatures are plotted with the seasonal curves of rumen bacteria. There is good agreement between all three curves, especially between daylight and bacteria which are both sine curves. This suggests that light and temperature may influence the seasonal fluctuation in bacteria. Light and temperature are believed to affect reproductive functions through their influence on the pituitary and thyroid glands, and light is known to affect coat changes in cattle and wool growth in sheep. The rumen environment could be influenced either by a direct effect on rumen temperature, which seems extremely unlikely in the present experiments, or by an effect on blood

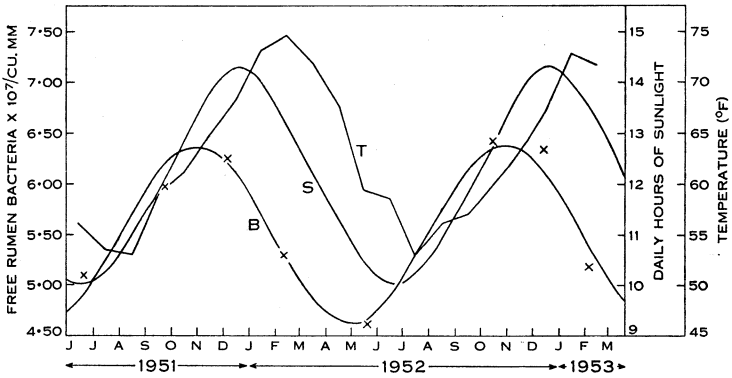

Fig. 4.—Seasonal fluctuations in rumen bacterial concentration (*B*), hours of sunlight (sunrise to sunset) (*S*), and mean monthly temperature (*T*). × Experimental values.

composition. Alterations in blood composition would presumably be reflected in saliva composition and the interchange of ions and water between rumen liquor and blood (Parthasarathy and Phillipson 1953). It is possible that fluctuations in rumen bacteria result from corresponding fluctuations in some substance or substances entering the rumen via the saliva or the rumen wall or leaving the rumen through the rumen wall.

Digestion and nitrogen metabolism experiments, to be reported elsewhere, were conducted concurrently with the seasonal bacterial determinations but, although there were significant period differences in the digestion of crude fibre, dry matter and nitrogen, and in nitrogen balances, these could not be related to fluctuations in the concentration of rumen bacteria.

V. Acknowledgments

Grateful acknowledgment is made to the Wool Research Trust Funds for financial assistance; to Professor E. J. Underwood, Director, Institute of Agriculture, for assistance and advice in the preparation of the manuscript; to Mr. R. J. Moir and

Mr. V. J. Williams for their numerous discussions and assistance in the experimental work; to Mr. N. Stenhouse, Division of Mathematical Statistics, C.S.I.R.O., for the statistical analysis of results, and finally, to Mr. J. Way and Mr. P. Clare for their care of the experimental animals.

VI. References

Bortree, A. L., Dunn, K. M., Ely, R. E., and Huffman, C. F. (1946).—*J. Dairy Sci.* **29**: 542.

Chance, C. M., Smith, C. K., Huffman, C. F., and Duncan, C. W. (1953).—*J. Dairy Sci.* **36**: 743.

Gall, Lorraine S., Smith, S. E., Becker, D. E., Stark, C. N., and Loosli, J. K. (1949).—*Science* **109**: 468.

Gall, Lorraine S., Stark, C. N., and Loosli, J. K. (1947).—*J. Dairy Sci.* **30**: 891.

Henneberg, W. (1922).—*Zbl. Bakt.* II Abt. **55**: 242.

Johnson, B. C., Hamilton, T. C., Robinson, W. B., and George, J. C. (1944).—*J. Anim. Sci.* **3**: 287.

Moir, R. J. (1951).—*Aust. J. Agric. Res.* **2**: 322.

Moir, R. J., and Williams, V. J. (1950).—*Aust. J. Sci. Res.* B **3**: 381.

Monroe, C. F., and Perkins, A. E. (1939).—*J. Dairy Sci.* **22**: 983.

Myburgh, S. J., and Quin, J. I. (1943).—*Onderstepoort J. Vet. Sci.* **18**: 119.

Parthasarathy, D., and Phillipson, A. T. (1953).—*J. Physiol.* **121**: 452.

Phillipson, A. T. (1942).—*J. Exp. Biol.* **19**: 186.

Phillipson, A. T., and McAnally, Rachel A. (1942).—*J. Exp. Biol.* **19**: 199.

van der Wath, J. G., and Myburgh, S. J. (1941).—*Onderstepoort J. Vet. Sci.* **17**: 61.

Williams, V. J., and Moir, R. J. (1951).—*Aust J. Sci. Res.* B **4**: 377.