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STUDIES ON ALTERATIONS IN THE RUMEN FLUID OF SHEEP, ESPECIALLY CONCERNING THE MICROBIAL COMPOSITION, WHEN READILY AVAILABLE CARBOHYDRATES ARE ADDED TO THE FOOD

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By

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Indigestion caused by an inexpedient diet is rather common in ruminants, principally in cattle. Due to the more intensive and extreme feeding nowadays, the frequency of such disorders seems to be increasing. The experience in this country indicates that the diet often has an unfortunate composition by containing too much readily fermentable carbohydrate as grain, roots, potatoes, molasses, skim milk, whey, etc.. As the supply of one or more of these ingredients frequently is subjected to seasonal variations, this leads to a corresponding variation in feeding. In addition, the change-over is often too abrupt, in only a few days the new feeding stuff is incorporated in the daily rations in maximum amounts. The result is a more or less serious indigestion, not seldom with a fatal outcome.

Ruminants differ from other mammals by converting an essential part of the fodder by microbial fermentation in the reticulum and rumen. As the ingested fodder, mixed with water and saliva, forms the culture medium for the flora and fauna in the rumen-reticulum sac, it is obvious that the microorganisms are influenced by the diet fed to the animal. That this is the fact is shown by a number of investigators, among others *Pounden* and *Hibbs* (1948), *Burroughs* et al. (1950), *Gall* et al. (1951, 1953), *Hungate* et al. (1952), *Williams* et al. (1953), *Bryant* and *Burkey* (1953), *Maki* and *Foster* (1957).

The purpose of these feeding experiments on sheep was especially to study the changes in the ruminal population when the food was supplemented with various readily fermentable carbohydrates. Cane sugar was the first carbohydrate to be tested and these investigations are described in the present paper.

MATERIALS AND METHODS

Adult, non-pregnant ewes equipped with permanent rumen fistulae were used as experimental and control animals. The fistulae were established surgically and closed by ebonite cannulae as described by *Quin* et al. (1938) and *Phillipson* and *Innes* (1939).

The animals were fed a good quality meadow hay to appetite and allowed free access to drinking water. The hay was given twice daily, viz. at 7 a.m. and 3 p.m.. Any leavings from the morning meal were taken away at 9 a.m. and the water was also withheld from that time to the next feeding.

Sampling the rumen contents was regularly carried out at 11 a.m. and when desirable, additional samples were withdrawn at appropriate intervals throughout the day. A 200 ml. milk-bottle was closed by a rubber plug through which were inserted two glass tubes. To these were fastened 1) a stout rubber tube connecting a suction-pump (an automobile-pump with inverted piston) and 2) a plastic tube leading into the rumen through the cannula. The last mentioned tube had a bore of 5 mm. and the free end was perforated in a length of about 10 cm.. Occasionally it was necessary to stiffen the plastic tube by a wooden stilet to penetrate very solid rumen contents. In order to avoid mixing with air and loss of carbondioxide, the bottle was filled to the top and closed immediately. This is why a second bottle was interposed between the sampling bottle and the pump. Thus, any overflowing would not reach the pump.

The experimental sheep were given their daily sugar rations the moment after sampling at 11 a.m.. The sugar, as a concentrated solution, was introduced through the rumen fistula. The control sheep was given the same volume of water.

The feeding experiments were either a) short period tests or b) long period tests. In case a) the sugar doses were increased rapidly, usually with a rise of 100 g. a day, until the animal sickened or died. In case b) the increase in sugar rations went

more slowly and the sugar feeding lasted for several weeks. Preceding every feeding experiment preliminary examinations of the rumen fluid were performed for about a fortnight.

Examination of the rumen fluid. The rumen contents were examined and tested as soon as possible after sampling, especially the electrometrical determination of pH and the cultivations were performed without delay.

Normal bright-field microscopy was used to examine the material in wet preparations unstained or stained with Lugols iodine, and in dried films stained by Gram's method.

As to wet preparations, 0,05 ml. of the specimen was examined under a 22 × 22 mm. coverslip. In unstained preparations particularly the motility of the protozoa was noted, while the amount of protozoa, yeastlike cells and eventually other iodophile microorganisms was recorded in the iodine-stained specimens. The Gram-stained films prepared after diluting the rumen fluid 1 to 5 were used for further examination of the bacterial flora.

No attempt was made to estimate the total number of protozoa, yeastlike cells, Gram-positive cocci and rods etc. per ml. rumen fluid, but the average number per field of view in preparations made identically, was registered. That should give an approximate idea of the fluctuations in the microbial population of the rumen fluid. For the sake of convenience, the amounts of the various microorganisms found in this way are in the tables marked by one or more "+". As to the protozoa, each + means 5 cells. The yeastlike cells are graded as follows: + = 5—10, ++ = 25, +++ = 50, ++++ = 100 cells, a. s. o. The number of bacteria are graded more at random.

Cultural methods. — Preparation of the basal medium. Cultivation of the samples was carried out parallel to the microscopical examination. The medium used was a rumen-liquor carbohydrate-agar similar to that recommended by *Heald* et al. (1953) for the cultivation of saccharolytic bacteria from the rumen of sheep.

The rumen liquor required for the basal medium was withdrawn from a hay-fed cow as a quantity of 1 to 2 liters was needed at a time and it is usually difficult to collect this amount from a sheep. Comparative tests performed previously with media prepared from rumen liquor of cow and sheep, indicated no difference in growth.

During filtration through Seitz sterilizing pads the pH of the clarified rumen liquor often changed, giving rise to a precipitate. In order to prevent this drawback the phosphate buffer used by *Heald et al.* was replaced by Mc' Ilvaines standard buffer. The composition of the buffer was: Na_2HPO_4 3,48 g., citric acid 1,92 g., distilled water 100 ml. — Two ml. buffer solution was added to 100 ml. rumen liquor. The rumen liquor now showed no sediment after filtration and storage for several months, and the medium prepared was clear and transparant.

The medium contained, per 100 ml.: 25 % casein hydrolysate solution, 2 ml., 1 % DL-tryptophane solution, 2 ml., 4 % soluble amylum solution, 4 ml. or 20 % glucose solution, 2,5 ml., 5 % agar solution, 30 ml., buffered rumen liquor, sterilized by filtration, 60 ml..

Apart from the rumen liquor, the other ingredients of the medium were sterilized by steaming.

Setting up the count. Sets of culture tubes, each tube containing 9 ml. melted medium and kept in a water bath at 45°C, were used for the cultivations. One ml. inoculum was taken from the rumen sample collected about 10 minutes previously and put aside to precipitate coarse fodder particles and serial dilutions (tenfold) were prepared directly in the culture medium. The series were incubated in CO_2-atmosphere at 38°C for 3 days, the cultures being examined daily. At the end the quantity of growth was determined by counting the colonies in the tube where the number was appropriate, usually in the range 25 to 200. If possible, the colonies in two subsequent dilutions were counted and equal weight given to each, the two counts usually showing good agreement.

After having estimated the growth quantitatively, the solid agar plug was transferred to a sterile Petri dish by gently heating the bottom of the culture tube in a Bunsen flame. Gram-stained films were prepared from all tubes showing growth. Agar plugs from tubes with dense growth were bisected and films prepared by scraping the surface, whereas several single colonies were picked out from each tube of the higher dilutions, including all the colonies from the last tube giving growth. In the same way well-separated colonies were isolated for subculturing and further identification of the growth.

RESULTS

Experiment 1. The daily sugar rations were increased rapidly as shown in table 1. In the same table are summarized some of the data obtained from the preliminary period as well as during the sugar feeding. All data from this experiment are related to rumen samples collected at 11 a.m., i. e. just before the daily administration of sugar.

Table 1. Data on the sheep in experiment 1.

Sucrose (grams)	pH of rumen	Protozoa	Yeastlike cells	Gram-pos. cocci	Gram-pos. rods	Colonies/ml. rumen fluid	
						Streptococci	Gram-pos. rods
0	6,8	+++	(+)	+	(+)	$4{,}6\times10^{6}$	0
0	6,8	+++	(+)	+	(+)	$1{,}2\times10^{6}$	0
0	6,9	+++	(+)	+	(+)	$3{,}9\times10^{6}$	0
0	6,8	++++	+	+	(+)	$3{,}5\times10^{6}$	0
100	6,9	+++	++	+	(+)	$4{,}5\times10^{6}$	0
200	6,8	++++	+	+	(+)	$5{,}0\times10^{6}$	0
300	6,9	+++++	+	+	(+)	$4{,}5\times10^{6}$	0
400	6,8	+++++	++	++++	(+)	$4{,}0\times10^{8}$	0
500	5,3	(+)	+++	+++	+++++	$5{,}0\times10^{7}$	$2{,}5\times10^{8}$
0	4,2	0	++	+	+++++	$< 1{,}0\times10^{5}$	$6{,}5\times10^{8}$

Marked changes in the physical properties, pH and microbial composition of the rumen contents were observed during the period of sugar feeding.

Concerning the physical properties no noticeable alteration occurred within the first three days as judged by the consistency, smell and colour of the rumen fluid. In the course of the next two days the consistency became thin and waterish and the samples showed rapid precipitation of coarse plant particles. At the same time the strong brown colour characteristic of rumen fluid from hay-fed sheep, changed to grayish and the normal smell was replaced by an increasing sourish odour. At the end of the experiment the rumen fluid could best be described as reminiscent of sour whey, suggesting a high concentration of lactic acid.

The pH of the samples kept quite unchanged until the fourth and fifth day when it suddenly dropped to 5,3 and 4,2 respectively.

The amount of yeastlike cells (Selenomonads, "Quin's oval") increased slightly during the period of sugar feeding and the

cells were more strongly iodophile than on the hay-diet. The sheep used in this experiment, however, had a rather small number of yeastlike cells before as well as during the sugar feeding.

As to the protozoal population, the rumen samples during the first three days of the sugar feeding disclosed vivaciously motile and strongly iodophile organisms in a number like that found in the preliminary period. In the next two days, however, coincident with the gross change of the rumen fluid, the protozoa disappeared completely. Only sporadic remnants, evidently in the act of decomposing, were all that were visible.

Gram-stained films of the rumen fluid revealed in the preliminary period a predominant association of Gram-negative microorganisms as usually found on hay feeding. This very mixed culture consisted of cocci and rods of highly different morphology, sarcinae, yeastlike cells, vibrios and spirals, Oscillospira guillermondii, etc., together with Gram-variable coccoids. The small population of Gram-positive bacteria was composed of mono- and diplo-cocci of somewhat variable size and shape and few short streptococci and small rods.

Through the first two days of the experiment no noticeable alteration in the ruminal flora was observed by direct microscopy, whereas the sample the following day revealed a considerable rise in the number of Gram-positive cocci. The fourth day the bacterial population had changed still more as Gram-positive rods and cocci were the overwhelmingly predominant organisms, the rods apparently outnumbering the cocci. The sample taken the day after the last administration of sugar revealed that the number of Gram-positive rods was of the same magnitude as the day before, whereas the Gram-positive cocci numerically had decreased considerably. Furthermore, the last two samples showed that the complex association of Gram-negative organisms originally present in abundance was now in the act of vanishing. Apart from yeastlike cells and sarcinae, the Gram-negative bacteria present seemed chiefly to be large coccoids occuring singly, in pairs and chains. The cells were about 2 microns in diameter, disc-like and closely packed when found in chains. In addition, the films revealed numerous faintly stained and indistinct particles judged to be debris of disintegrating microorganisms.

The Gram-positive rods which flourished in the rumen the last two days of the experiment were of uniform morphology.

They were straight or slightly curved rods with rounded ends, 0,8—1,0 × 1,5—2,5 microns, more rarely up to 3—4 microns in length, usually occuring singly but short chains were also seen. Spores were never observed, nor motility in fresh preparations.

By examination of an additional sample withdrawn in the evening the last day of the experiment, the rods presented a more variable Gram-reaction. Apart from being strongly Gram-positive, a large number showed uneven and granular staining, and fully Gram-negative rods of the same morphology were noticed as well. Further, the rods showed more variation in size, ranging from 1,5 to 6—8 microns in length.

The cultures set up during the preliminary period gave little variation in the numbers of colonies developing. Referring to Table 1, the counts varied from $1{,}2 \times 10^6$ to $4{,}6 \times 10^6$ per ml. rumen fluid. The growth was evenly distributed in the medium and well separated colonies seemed to be of a uniform type in the form of compact, lenticular, whitish colonies. The size of the colonies increased throughout the series, reaching a diameter of about 2 mm. in the last tube showing growth. The growth was very rapid, the cultures being usually fullgrown in 18—20 hours. Apart from the first 2—3 tubes in each series, no gas production was observed in the cultures.

Microscopic examination of Gram-stained films of individual colonies revealed, with very few exceptions, exclusively small Gram-positive cocci in pairs and short chains. Smears prepared from the dense growth in the first 2—3 tubes in the series showed, as a rule, a mixed bacterial flora.

The cultures the first two days after the dosing of sugar was started, did not show any difference in growth, quantitatively or qualitatively, from that obtained in the preliminary period. On the other hand, the colony count the next day showed a hundredfold increase, amounting to 4×10^8 per ml. rumen fluid. The morphology of the colonies seemed to be as noticed previously. Microscopy of a large number of single colonies revealed exclusively Gram-positive cocci, whereas smears from the first tubes in the series in addition disclosed short Gram-positive rods up to dilution 1:1000.

The cultures the fourth day were evidently presenting two morphologically different kinds of colonies. The large lenticular colonies observed formerly were still present but the count had decreased to 5×10^7 per ml. rumen fluid. The new colony type

encountered appeared as small round colonies the number of which was about fivefold that of the lenticular type, viz. $2{,}5 \times 10^8$. This tendency of the growth towards replacing the large colonies originally present in the cultures by minute colonies, was still more pronounced the next day, i.e. the last day of the experiment. The large colonies could now only be seen in the lower dilutions, the count being less than 1×10^5, whereas the number of small colonies showed further rise to $6{,}5 \times 10^8$. The cultures inoculated

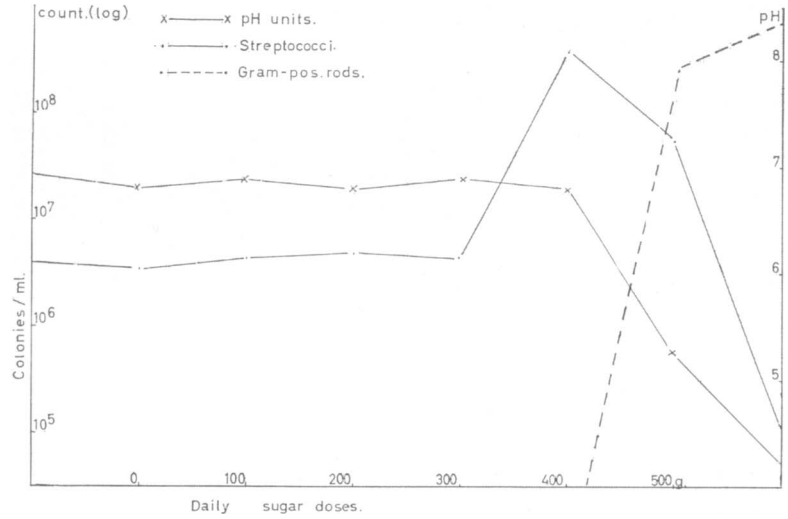

Fig. 1. Fluctuations in rumen pH and colony counts in experiment 1.

with rumen contents withdrawn in the evening this day, gave exclusively small colonies.

Microscopic examination of the cultures these last two days of the experiment showed as before that the large colonies contained Gram-positive cocci. The small colonies consisted of Gram-positive rods of similar morphology to those found in the rumen fluid used as inoculum. The cultures set up the last evening disclosed entirely Gram-positive rods, whereas Gram-positive cocci were not found at all.

The fluctuations in rumen pH and colony counts (log) of streptococci and Gram-positive rods per ml. rumen fluid is shown graphically in figure 1.

The cultivations in this experiment showed that the medium employed provided very good growth to the saccharolytic streptococci whereas the Gram-positive rods were growing less vigorous-

ly. Although the medium was obviously not optimal for the rods, it was still used in the following experiments because the difference in colony appearance facilitated the differentiation in cocci and rods.

Experiment 2. In this experiment too the daily amounts of sugar given to the animal were increased rapidly as will be seen from table 2.

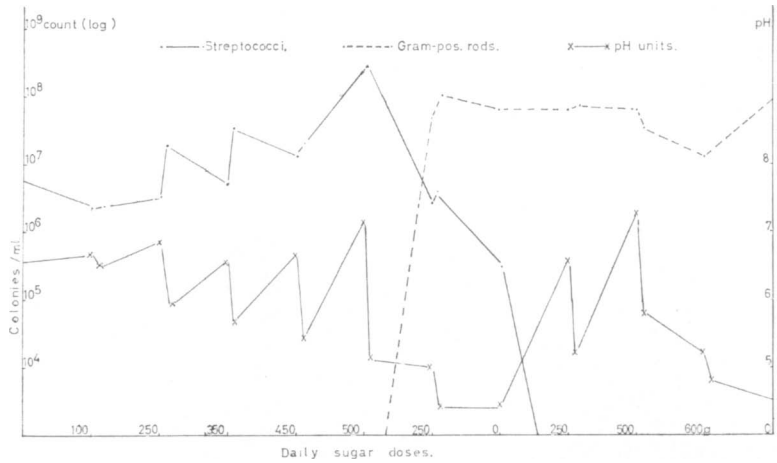

Fig. 2. Variations in ruminal pH and colony counts in experiment 2.

After reaching a dosage of 500 g. the animal sickened, showing depression and diarrhoea and completely suppressed appetite. In order to see how a reduction and a temporary discontinuance in the administration of sugar would influence the indigestion induced, the sheep the next two days was given 250 g. sugar and nothing at all, recpectively. The day after this stop in the sugar feeding the animal was looking better and started eating again, and the dosing of sugar was recommenced.

In addition to the usual sampling just before the daily administration of sugar, which was called 0 hour, samples were regularly withdrawn 3 hours later and sometimes 5 and 7 hours after the sugar was given.

Results obtained in this experiment are listed in table 2 and shown graphically in figure 2.

On the whole, the rumen fluid underwent similar changes in physical properties, pH and microbial composition as in experiment 1.

Table 2. Dosing of sugar and data obtained in experiment 2.

Sucrose (grams)	pH of rumen	Protozoa	Yeastlike cells	Gram-pos. cocci	Gram-pos. rods	Colonies/ml. rumen fluid	
						Streptococci	Gram-pos. rods
0	6,6	++++++++	+	+	(+)	$6{,}1 \times 10^6$	0
0	6,6	+++++++++	+	+	(+)	$7{,}6 \times 10^6$	0
0	6,6	++++++++	+	+	(+)	$5{,}3 \times 10^6$	0
100	6,7	++++++++	+	+	(+)	$2{,}2 \times 10^6$	0
	6,5*					$2{,}5 \times 10^6$	0
250	6,8	+++++	+	+	(+)	$2{,}9 \times 10^6$	0
	5,9*	+++++	++	++	(+)	$1{,}9 \times 10^7$	0
350	6,6	++++++++	+	+	(+)	$3{,}9 \times 10^6$	0
	5,7*	++++++	+++			$2{,}9 \times 10^7$	0
450	6,7	++++++++	+	++	(+)	$1{,}5 \times 10^7$	0
	5,4*	++++	++	++	(+)	$2{,}1 \times 10^7$	0
500	7,2	(+)	+	++++	+	$2{,}5 \times 10^8$	0
	5,1*	+++	+++	+++++	+	$2{,}7 \times 10^8$	0
250	5,0	0	++	++	+++++	$2{,}2 \times 10^6$	$5{,}0 \times 10^7$
	4,4*	0	+	++	+++++	$3{,}5 \times 10^6$	$1{,}0 \times 10^8$
0	4,4	0	+	++	+++++	$5{,}0 \times 10^5$	$7{,}5 \times 10^7$
250	6,6	0	+	++	+++++	$< 1{,}0 \times 10^5$	$7{,}0 \times 10^7$
	5,2*	0	(+)				$7{,}5 \times 10^7$
500	7,3	0	(+)	++	+++++	0	$7{,}0 \times 10^7$
	5,8*	0	(+)				$3{,}2 \times 10^7$
600	5,2	0	(+)	++	++++	0	$1{,}5 \times 10^7$
	4,8*	0	0				
0	4,4	0	0	+	+++++	0	$9{,}0 \times 10^7$

* 3-hour samples.

Concerning the physical properties a slightly fecal odour was noticed in addition to the sourish smell at the end of the experiment. At the same time a moderate number of Gram-negative coliform rods was found in the smears and cultures.

The pH-values in the 0-hour samples did not differ from those obtained in the preliminary period (6,6—6,8) until the introduction of 450 g. sugar which led to the rumen fluid becoming slightly alkaline (7,2), in spite of the 3-hour samples during the same period were showing a steadily increasing acidity. A further increase to 500 g. resulted in a fall in pH to 5,0 and it went on dropping to 4,4 after the following dosing of 250 g. sugar. After discontinuing the sugar feeding for one day the ruminal pH rose to normal level (6,6) and continued rising to 7,3 after the addition of 250 g. sugar. The second feeding of 500 g. sugar did not cause so rapid depression in pH as the same amount did the first time, but on the other hand, the pH continued to decrease throughout the day. This different effect of the same amount of sugar may in part be due to the alteration in the ruminal flora, the streptococci being replaced by Gram-positive rods (cp. Fig. 2). A similarly protracted reduction in pH was noted after feeding 600 g. sugar the last day of the experiment.

Some data obtained by the microscopic examination of the rumen samples are given in table 2, column 3 to 6. As will be seen, the rumen fluid in the preliminary period was very rich in protozoa, but poor in yeastlike cells. About three fourth of the protozoal fauna consisted of small oligotrich ciliates and the remaining part were mainly large holotrichs. The last mentioned ciliates were more or less iodophile, whereas the oligotrichs showed negative or weak iodine reaction. The bacterial population showed, as usual, a preponderance of Gram-negative organisms and a spare Gram-positive flora, consisting of cocci and a few rods.

During the first three days of the experiment, no marked difference in the number of protozoa was noticed. In the 0-hour samples, the holotrichs only showed positive iodine reaction, whereas the other protozoa too were iodophile 3 hours later. On the other hand, the protozoa were apparently killed when the pH dropped to about 5,5. The third day of the experiment, their motility appeared normal in the 0-hour sample, 3 hours later (pH 5,7) it was distinctly depressed, and the following day

(pH 5,4) motile protozoa were hardly observed. Two days later protozoa were no longer found in the rumen samples.

The 0-hour samples did not show any noticeable variation in the numbers of yeastlike cells until the last two-three days of the experiment when only remnants of such cells were found. However, each administration of sugar seemed to bring about a temporary rise in the amount of yeastlike cells, since the 3-hour samples were showing a larger density of distinctly iodophile cells.

Examination of Gram-stained films during the experimental period disclosed similar changes in the bacterial flora as those encountered in experiment 1. The Gram-positive cocci did not show any observable rise in numbers in the 0-hour samples until the third day and this increase culminated the next day. The coccal population, however, remained throughout the experiment on a level above that found in the preliminary period. As to the 3-hour samples a multiplication of Gram-positive cocci was already observed the second day of the experiment. Coincident with the peak in the coccal population a moderate increase in the number of Gram-positive rods was found in the rumen contents. During the following day these rods were evidently multiplying enormously as they were the overwhelmingly predominant organisms in the rumen fluid the next day and they apparently kept this level throughout the experiment except for the second last day when the films revealed a smaller density. These rods seemed morphologically to be of a uniform type. They were non-motile, slender rods with rounded ends, straight or slightly curved, ranging from 1,5 to about 8 microns in lenght, and 0,7—0,8 microns in diameter, occurring singly or in pairs. They were mostly distinct Gram-positive, but irregularly stained rods showing granulation and uneven staining were also observed.

Along with the development of the Gram-positive flora the Gram-negative bacterial association originally present was strongly reduced. With the exception of a relatively small number of coliform rods, mainly indistinct remnants of other Gram-negative organisms were found at the end of the experiment.

As a whole, the results of the cultivations (see table 2, column 7 and 8, and figure 2), were in fairly good agreement with the observations made by the direct microscopic examination of the rumen samples. During the preliminary period and the first four days of the sugar feeding the cultures gave growth only to

streptococcal colonies. The colony counts in the first two 0-hour cultures set up after the sugar feeding was started were within the same range as in the preliminary period in spite of a marked increase in the 3-hour culture the second day. The next two days both 0-hour and 3-hour cultures gave counts higher than normal, the rise reaching its peak the fourth day. The following day the cultures presented colonies of two different types. The predominating flora consisted of minute colonies, whereas the large lenticular colonies had decreased in number, the counts indicating a fall in 21 hours from $2{,}7 \times 10^8$ to $2{,}2 \times 10^6$ per ml. rumen fluid. This numerical reduction in streptococcal colonies continued the following two days, the counts being 5×10^5 and less than 1×10^5, respectively. The remaining three days of the experimental period streptococci were only found by microscopic examination of films prepared from the dense growth in the first few tubes of the series. This sparse growth of streptococci did not seem to correspond with the results obtained by direct microscopy of the rumen fluid, showing a coccal population rather larger than normal. This apparent disagreement may be due to the fact that most of the cocci in the rumen were no longer viable. Large colonies resembling the streptococcal colonies were found in the cultures these last days, counts ranging from 2×10^5 to 4×10^5 per ml. rumen fluid, but these colonies proved to be Gram-negative coliform rods.

The minute colonies which suddenly flourished in the cultures the fifth day, giving the counts 5×10^7 and 1×10^8 per ml. rumen fluid, kept this level throughout the experiment except the second last day when a temporary drop to $1{,}5 \times 10^7$ was found. This fall may be due to the consumption of water this day whereas the animal the preceding two days and the following day did not drink at all. These colonies consisted of Gram-positive slender rods, usually showing irregular staining. The rods resembled those found in the rumen fluid, but were more variable in size, ranging from 1,5 to about 25 microns in length, the long forms often being strongly curled. Rods of this morphology and staining reaction were also found in tubes of lower dilutions the day before the minute colonies appeared in the cultures, indicating a number less than 1×10^5 per ml. rumen fluid.

Throughout this experiment rumen fluid from the control animal was investigated parallel to that of the experimental

animal. The results obtained were fairly invariable and the 3-hour samples did not differ from the 0-hour samples.

Experiment 3. The experimental animal was the same as that used in experiment 2 three months earlier and the doses of sugar given were approximately as in the previous experiment. Rumen samples were withdrawn just before each administration of sugar except the second day when the animal was not sampled at all. Data obtained are given in table 3 and shown graphically in figure 3.

Table 3. Data on the sheep in experiment 3.

Sucrose (grams)	pH of rumen	Protozoa	Yeastlike cells	Yeasts	Colonies/ml. rumen fluid	
					Streptococci	Gram-pos. rods
0	6,8	++	++	0	$1,5 \times 10^6$	0
0	6,9	++	+++	0	$2,7 \times 10^6$	0
0	6,7	++	+++	0	$3,0 \times 10^6$	0
100	6,8	+++	++	0	$2,5 \times 10^6$	0
200						
300	5,3	(+)	(+)	0	$3,8 \times 10^7$	$3,0 \times 10^6$
350	4,0	0	(+)	$1,0 \times 10^4$	$3,0 \times 10^3$	$7,0 \times 10^7$
400	4,0	0	0	$1,2 \times 10^7$	$3,0 \times 10^2$	$2,1 \times 10^7$
0*	3,9	0	0	$1,7 \times 10^7$	$1,0 \times 10^2$	$1,6 \times 10^7$

* The sheep died in the night and was sampled post mortem.

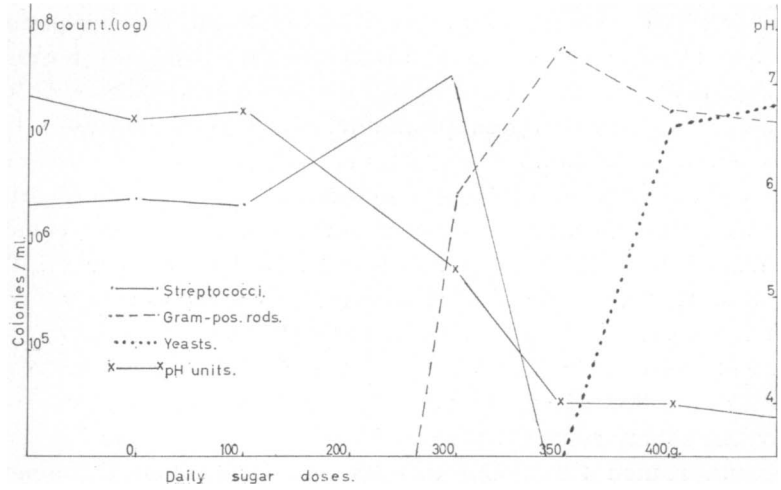

Fig. 3. Fluctuations in rumen pH, colony counts of streptococci and Gram-positive rods and the amount of yeasts in experiment 3.

In the preliminary period the rumen fluid contained far less protozoa but more yeastlike cells than prior to the preceding experiment, otherwise no noticeable difference was found.

The feeding of sugar brought about similar changes in the rumen contents as those observed in experiment 2, but they were induced by much smaller amounts of sugar. The addition of 200 g. sugar had approximately the same effect as 500 g. in the former experiment: the rumen fluid became greyish with slightly sourish smell, the pH fell to 5,3, protozoa and yeastlike cells were on the point of disappearing, the number of Gram-positive cocci showed a marked rise and a fairly large population of Gram-positive rods appeared in the rumen fluid.

Microscopic examination of the rumen fluid the following day revealed a microorganism which was never before observed in the samples. This organism appeared as oval cells about 4—5 $\times$ 6—8 microns in size, occuring singly, in short chains and small clusters. The cells were non-motile, showed positive iodine and Gram reactions and budding cells were frequently observed. On the basis of these characters the organism was supposed to be a yeast. Counting carried out by Breed's method indicated a number of approximately 10.000 cells per ml. rumen fluid. Obviously the cells were multiplying vigorously in the following 24 hours as the next sample gave a count of about 12 million cells per ml. The last sample taken post mortem the next day indicated a number within the same range, viz. $1{,}7 \times 10^7$. Especially in fresh preparations the cells the last two days frequently occurred in chains and clusters containing 10 to 15 and 50 to 60 organisms respectively. At this time the sourish odour of the rumen contents was less pronounced and it smelt more like yeast-fermented jam.

A great number of large polygonal cells, presumably originating from the stratified squamous epithelium in the rumen-reticulum also appeared in the last two samples collected. The cells were partly containing nuclei and occurred singly or in small flakes.

The increase in the number of Gram-positive cocci after the second addition of sugar was followed by a sharp fall the next day, the colony counts these two days were $3{,}8 \times 10^7$ and 3×10^3 per ml. rumen fluid, and this decrease continued throughout the experiment.

The Gram-positive rods which appeared simultaneously with

the rise in the coccal population reached, judging by the colony counts, their highest number (7×10^7) the next day, as there was later a tendency for counts to be lower, but they were still within the same logarithmic range. The rods were of similar morphology as those encountered in experiment 2.

In agreement with the previous experiments the Gram-negative flora was reduced during the period of sugar feeding. At the end of the experiment the Gram-positive rods and yeasts were completely outnumbering the Gram-negative bacteria which were

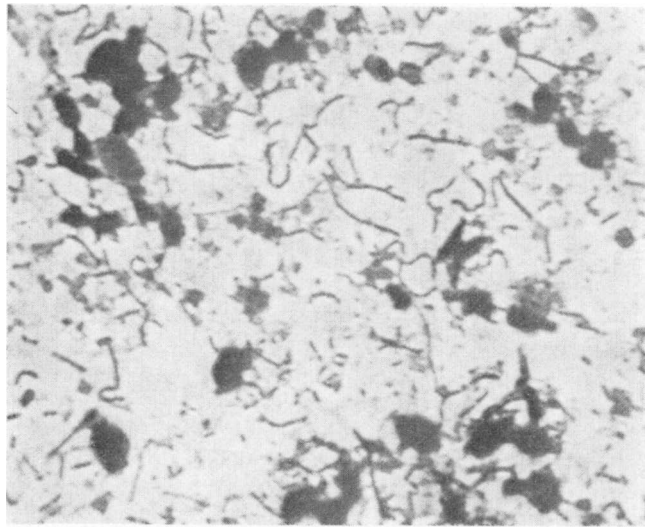

Fig. 4. The rumen flora at the end of experiment 3, mainly consisting of yeasts and Gram-positive rods. × 800.

chiefly faintly stained and undistinct cocci and coccoids. Figure 4 shows a microphoto of a Gram-stained film prepared from the rumen fluid at that stage.

Experiment 4. The sugar feeding in this experiment was extended over a period of four weeks, starting with daily sugar doses of 50 g. and adding 25—50 g. every third day until a daily amount of 450 g. was reached. Rumen fluid was collected once a day immediately before the sugar was given.

During the first fourteen days of the experimental period, i.e. until the first dose of 250 g. sugar, no change in the rumen fluid was noted either macroscopically, by microscopic examination or by cultivation. On the other hand, the next two doses

of 250 g. sugar caused a sudden alteration in the ruminal flora and fauna. The protozoa and yeastlike cells which all the time had been present in quite large amounts, disappeared almost entirely and the streptococcal colony count increased from 6 to 210 million per ml. rumen fluid. During the following two days the streptococcal count rose further to 310 million followed by a decrease to 130 and 22 million the next two days. This fall continued and at the end of the experiment the number was about 200.000 per ml. rumen fluid.

Coincident with the peak in the number of streptococci, Gram-positive rods commenced flourishing in the rumen fluid, the cultures showing a colony count of about 10.000 per ml.. These rods continued to increase in number as the streptococci were disappearing. Microscopic examination of the rumen samples the last four to five days of the experiment revealed a ruminal flora which was completely dominated by Gram-positive rods, the rest mainly consisting of Gram-negative coliform rods and Gram-positive cocci. The cultures set up these days indicated a colony count of Gram-positive rods of about 40 million per ml. rumen fluid.

The rods encountered in the rumen the last ten days changed morphology as the experiment went on. At first they appeared as short rods approximately 2—3 microns in length, frequently growing in long chains and showing a strong Gram-positive reaction. Later on they were extremely pleomorphic, often occurring as long filaments with very variable Gram-staining.

At the end of the experiment Gram-positive oval cells like those encountered in experiment 3 and judged to be yeasts appeared in the rumen contents in numbers amounting to about 10 million per ml.. At the same time polygonal epithel-like cells were also found in relatively large amounts.

As to the reaction of the rumen contents during the period of sugar feeding, a slight drop in pH was observed at the end of the first week (from 6,7 to 6,2), whereas the pH-values the following fortnight constantly kept a level of 6,8—6,9. At the beginning of the fourth weak the rumen fluid became slightly alkaline (7,1—7,3) followed by a sharp fall to 4,9, 4,3 and 3,8 the last three days. Ruminal pH post mortem was found to be 3,4.

Regarding the physical properties of the rumen fluid, changes similar to those described in experiment 3 were observed simultaneously with the alterations in the ruminal flora.

Experiment 5. This experiment was started by dosing 50 g. sugar daily and adding 50 g. every third day until a dosage of 200 g. which did not bring about any noticeable change in the rumen contents. From now the animal was given 200 g. daily in order to see the effect of a constant dosing of this amount of sugar. Rumen samples were withdrawn once a day just prior to the administration of sugar. Briefly described the following results were obtained.

There was observed no change in the rumen fluid the first four days after the dosing of 200 g. sugar had commenced. As in the first period of this experiment the rumen samples were still rich in protozoa and yeastlike cells, the streptococcal colony counts varied within very narrow limits (from 1 to 3 million per ml. rumen fluid) and the pH-values were within the range 6,8 to 7,2. The fifth administration of 200 g. sugar was followed by a rise in pH to 7,9, a ten-fold increase in the streptococcal colony count and a marked reduction in the number of protozoa and yeastlike cells. After the next dosing the pH fell to 4,3, protozoa could hardly be observed any longer and the yeastlike cells appeared deformed and agglutinated in clusters. Gram-stained films disclosed that Gram-positive rods and cocci were the predominating bacteria in the rumen and the cultures indicated a colony count of approximately 80 million of each per ml. rumen fluid.

Coincident with this rapid alteration in the rumen contents the animal got markedly depressed and the appetite was completely lost. Therefore the administration of sugar was discontinued but any treatment of the animal was not introduced. Already the following day the pH of the rumen was on the normal level and the animal took a little hay and after two additional days it had apparently recovered, showing good appetite. In addition, examination of the rumen fluid carried out as previously revealed that the microbial population of the rumen had returned to normal.

Identification of the Gram-positive ruminal flora. For further examination of the Gram-positive flora which flourished in the rumen during the feeding experiments well-separated colonies were isolated for subculturing and classification. All isolates of streptococci were amylolytic and proved to be Streptococcus bovis

and varieties. The Gram-positive rods proved to be various species of lactobacilli and the yeasts presumably belonged to the genus Candida. A more detailed description of the characteristics of the isolated microorganisms will be given in a following paper.

CONCLUSION

Under the experimental conditions used it was found that cane sugar introduced into the rumen of sheep maintained on a hay diet leads to a complete change in the microbial population of the rumen. This change is characterized by the development of a predominating Gram-positive flora consisting of amylolytic streptococci, lactobacilli and sometimes yeasts, whereas the ruminal fauna and Gram-negative flora originally present are destroyed or strongly reduced. The protozoa were apparently killed when rumen pH fell to about 5,5.

The development of the predominating Gram-positive flora evidently followed a certain pattern. The first stage was a marked rise in the amounts of streptococci but only transiently as, judged by the colony counts, the streptococcal flora was later depressed far below the initial level or disappeared entirely. In some of the experiments this sparse growth or lack of growth was apparently in disagreement with the abundant coccal flora found by direct microscopy of the rumen contents but this may be due to the cocci being no longer viable. Coincident with or following the peak in the streptococcal counts the lactobacilli commenced to flourish in the rumen, reaching their maximum amounts in one to two days and keeping this level throughout the experiment. In cases where yeasts turned up in the rumen, this occurred after the population of lactobacilli was established.

By giving glucose or grain in excess as a single dose to sheep, *Hungate et al.* (1952) found Strept. bovis most commonly responsible for the acid indigestion induced, whereas lactobacilli were supposed to be of minor importance. These results are in accordance with the first stage of changes in the rumen obtained in the present experiments, showing Strept. bovis and variants to be the predominant organisms, whereas lactobacilli, if present, were usually found in moderate numbers. The suggestion that lactobacilli may be important in producing high acidity in the rumen was evident from the second phase of rumen changes induced by continuing the sugar feeding. As a rule, this brought

about a ruminal flora completely dominated by lactobacilli giving a rumen pH in the range 3,8 to 4,5. This high acidity was apparently unfavourable to the streptococci which were killed or at least markedly inhibited in their growth. Clinical cases of indigestion showing a corresponding "lactobacillosis" of the rumen are repeatedly observed by the author in sheep as well as in cattle.

A significant feature of the experiments was that the changes in the microbial composition of the rumen did not develop gradually from day to day throughout the experimental period but set in rather suddenly when sugar had been given for some time. This fact was especially marked in the long-period experiment with slow increase in sugar feeding which gave no noticeable alteration during the first fortnight followed by a complete change in the rumen population in the next two days. The feeding of a constant ration of sugar apparently worked in the same way. Presumably one may conclude from these results that ruminants under practical conditions may be kept for some time on a ration too rich in carbohydrates without showing any symptoms of rumen disturbances. However, such a feeding breaks down the original stability of the rumen function, turning it into a labile state which is more sensitive to the inexpedient feeding or to minor changes in the ration.

As to the reaction of the rumen contents, the daily samples did not show any lowering in pH until the lactobacilli had grown up, which, as a rule, caused a marked drop. A large streptococcal flora alone did only cause a temporary fall in pH, lasting from a few to several hours depending on the amount of sugar given. In addition, at the time when the streptococcal flora was at its highest the transitory drop in pH was frequently followed by an alkaline reaction with pH-values up to 7,9. This fact may be of some interest from a clinical point of view. Clinical cases of rumen disturbances induced by intake of food rich in readily fermentable carbohydrates may present alkaline rumen contents when examined several hours after the feed is consumed, inviting to treatment with acid. Obviously that would be contraindicated as there in reality is an acid indigestion with, judging from the experiments on sheep, strongly acid urine.

That there may be a different resistance to sugar in one and the same animal maintained on the same hay diet is indicated by the results in experiments 2 and 3 which revealed that a daily

dose of 200 g. sugar in the latter had a corresponding effect on the rumen as 500 g. in the former. As far as could be judged by examination of the rumen fluid in the preliminary periods, the most striking difference was related to the amount of protozoa which in the first experiment was 4 to 5 times that found in experiment 3. This fact may at least in part explain the different resistance to sugar as a large population of protozoa will bring about that less sugar is subjected to bacterial fermentation with the production of acids.

The two cases where large amounts of yeasts were found in the rumen towards the end of the experiment had a fatal outcome. The rumen contents of both animals possessed an odour of alcoholic fermentation but unfortunately no analysis on alcohol was undertaken. Pure cultures of the yeast in question fermented sucrose very rapidly in vitro and yielded an alcohol concentration in worth of approximately 3 vol. %. Compared with the experiments with lambs on a glucose diet described by *Cunningham & Brisson* (1955) who found a rumen and blood alcohol content up to 0,616 and 0,455 % respectively, resulting chiefly from bacterial alcoholic fermentation in the stomach, one is inclined to assume that an alcohol intoxication was superimposed the acid indigestion in these two experiments.

In the two experiments just mentioned, epithelial cells obviously originating from the ruminal mucosa were found in abundance in the rumen contents the last days, indicating a considerable desquamation of rumen epithelium and supposed to be a symptom of acute rumenitis. This assumption was verified at autopsy which disclosed edema, hyperemia, hemorrhages and in part devillation of the ruminal mucosa. These findings correspond with the gross lesions by acute rumenitis in cattle induced experimentally by feeding barley in excess as described by *Jensen et al.* (1954). Probably this injury of the ruminal mucosa caused increased absorption of toxic substances from the rumen contents and thereby aggravated the condition. On the other hand, it appeared from experiment 5 that an indigestion with severe clinical symptoms and extremely altered rumen contents was spontaneously cured in a couple of days after discontinuing the sugar feeding. In this case neither epithelial cells nor yeasts were observed in the rumen.

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SUMMARY

The feeding of cane sugar in excess to sheep maintained on a hay diet led to indigestion with marked changes in the microbial population, pH and physical properties of the rumen fluid.

The microbial alterations were characterized by the development of a predominating Gram-positive flora in this sequence: amylolytic streptococci, lactobacilli and eventually yeasts. The protozoa were killed and the Gram-negative flora strongly reduced. These changes in the rumen microorganisms did not develop gradually from the very start of the experimental period but turned up rather abruptly when sugar had been given for some time.

A large streptococcal flora only caused a temporary fall in rumen pH, whereas the succeeding population of lactobacilli induced a constant high acidity in the rumen (pH 3,8—4,5). As a rule, an increase to alkaline reaction preceded the final drop in pH.

The physical properties (consistency, smell and colour) of the rumen fluid changed coincident with the alterations in the ruminal flora.

A highly different resistance to sugar was observed in one and the same animal maintained on the same diet.

Large amounts of epithelial cells were found in the rumen fluid when the sugar feeding resulted in a rumenitis.

An extremely altered rumen fluid may return to normal in a couple of days when the causative factor is eliminated.

ZUSAMMENFASSUNG

Untersuchungen über Änderungen der mikrobiellen Zusammensetzung des Pansensaftes beim Schaf bei Zuschuss von leichtverdaulichen Kohlehydraten zum Futter. I. Rohrsucker.

Zuschuss von Zucker an Schafe bei Heufütterung führte zu Indigestion mit ausgesprochenen Änderungen der mikrobiellen Zusammensetzung, des pH-Wertes und der physikalischen Eigenschaften des Pansensaftes.

Die mikrobiellen Änderungen bestanden in der Entwicklung einer dominierenden Gram-positiven Flora von amylolytischen Streptokokken, Lactobazillen und in einzelnen Fällen von Hefepilzen in der genannten Reihenfolge. Die Pansenfauna und die Gram-negative Flora zeigten Destruktion oder starke Reduktion. Diese Änderungen der mikrobiellen Zusammensetzung des Pansens entwickelten sich nicht gradweise während der ganzen Versuchsperiode, sondern entstanden ziemlich plötzlich nach kürzerer oder längerer Zeit während der Versuche.

Eine grosse Streptokokkenmenge im Pansensaft verursachte gewöhnlich nur eine vorübergehende Senkung des pH-Wertes, während die Lactobazillen einen dauernd niedrigen pH-Wert (3,8—4,5) ergaben. In der Regel stieg die Reaktion des Pansensaftes zu alkalischen Werten umittelbar vor der endgültigen Senkung im pH-Wert.

Die physikalischen Eigenschaften (Konsistenz, Geruch und Farbe) des Pansensaftes veränderten sich gleichzeitig mit den mikrobiellen Änderungen.

Ein grosser Unterschied in der Toleranz gegenüber Zucker wurde bei ein und demselben Tier unter gleichen Fütterungsverhältnissen festgestellt.

In Fällen, in welchen die Zuckerfütterung die Entstehung einer Rumenitis verursacht hatte, wurden zahlreiche Epithelzellen im Pansensaft gefunden.

Ein hochgradig veränderter Pansensaft könnte wenige Tage nach Abbruch der Zuckerfütterung völlig normale Verhältnisse zeigen.

SAMMENDRAG

Undersøkelser over endringer i vomsaftens mikrobielle sammensetning hos sau ved tilskudd av lettfordøyelige kullhydrater til fôret. I. Rørsukker.

Tilskudd av sukker til sauer på høyfôring resulterte i indigestion med uttalte endringer i vomsaftens microbielle sammensetning, pH og fysikalske egenskaper.

De mikrobielle endringer bestod i framvekst av en dominerende Gram-positiv flora av amylolytiske streptokokker, lactobaciller og enkelte ganger gjærsopp i nevnte rekkefølge. Vomfaunaen og den Gram-negative flora ble destruert eller sterkt redusert. Disse forandringer i vompopulasjonen utviklet seg ikke gradvis gjennom hele forsøksperioden, men oppstod relativt plusselig kortere eller lengere tid ut i forsøkene.

En stor streptokokkmengde i vomsaften forårsaket vanlig bare et forbigående fall i pH mens lactobacillene ga en vedvarende lav pH (3,8—4,5). Som regel steg vomsaftens reaksjon til alkaliske verdier umiddelbart før det endelige fall i pH.

Vomsaftens fysikalske egenskaper (konsistens, lukt og farge) endret seg samtidig med de mikrobielle forandringer.

Det ble registrert stor forskjell i toleransen overfor sukker hos et og samme dyr under like fôringsforhold.

Det ble funnet rikelig med epitelceller i vomsaften i tilfeller hvor sukkerfôringen hadde forårsaket oppståelsen av en rumenitis.

En høygradig endret vomsaft kunne vise helt normale forhold et par-tre dager etter at sukkerfôringen var avbrutt.

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