APPLIED MICROBIOLOGY, Dec. 1972, p. 871-877

Vol. 24, No. 6
Printed in U.S.A.

Effect of Low-Roughage Diets on the Microflora and Lipid Metabolism in the Rumen

M. J. LATHAM, J. E. STORRY, AND M. ELISABETH SHARPE
National Institute for Research in Dairying, Reading RG2 9AT, England

Received for publication 3 August 1972

Changing the diet of five lactating cows and one nonlactating cow from high to low roughage induced milk fat depression in the lactating cows and altered the composition of the rumen microflora. While the numbers of lactic and propionic acid-producing bacteria increased, the numbers of *Butyrivibrio* spp. decreased. The numbers of lipolytic bacteria and the in vitro lipolytic activity of the rumen fluid were also decreased, as was the extent of hydrogenation of linoleic and linolenic acids combined in soybean oil incubated in vitro with rumen fluid. It is suggested that among the bacterial population in the rumen the vibrios, which were adversely affected by the low-roughage diets, may contribute significantly to both lipolysis and hydrogenation in the rumen.

Although most diets of ruminants normally contain high proportions of polyunsaturated acids as phospholipids and galactoglycerides, only small amounts of these acids are directly transferred to adipose tissue or to milk since most of them are metabolized to more saturated derivatives by the rumen microflora (31) before being absorbed from the small intestine. Hydrolysis of the complex dietary lipids is necessary before isomerization and hydrogenation of the fatty acids can take place (15). When cows are fed low-roughage diets, particularly those which depress milk fat secretion, the changes in the fatty acid composition of lipids of milk (25), blood plasma (9, 21, 27), digesta (29, 33), and adipose tissue (30) suggest that hydrogenation in the rumen of dietary unsaturated acids may be reduced. Reductions in the numbers of ciliate protozoa have been associated with reduced hydrogenation (6), and lower numbers of these organisms and of the bacterium *Butyrivibrio fibrisolvens*, which also actively hydrogenates (23), are known to occur with low-roughage rations (4, 18, 24).

In this investigation the effect of low-roughage diets, including milk fat-depressing diets, on the composition of the microflora and the metabolism of feed lipids in the rumen have been examined more fully. The effect of these diets on the rumen fermentation are reported elsewhere (Latham, Sutton, and Sharpe, *in press*).

MATERIALS AND METHODS

Animals and their management. Rumen contents from four lactating Friesian cows with permanent rumen fistulas were used for in vitro studies on hydrogenation. Their diet was progressively changed from a control of high roughage (8 kg of hay and 10 kg of concentrate cubes, 20% dry weight as crude protein, daily) to a milk fat-depressing low-roughage diet in which flaked maize was substituted for part of the hay and concentrates until the milk fat content was depressed to less than 2%. The final proportions (in kilograms) of hay, concentrates, and flaked maize varied for each cow and were respectively 1, 1, 9 (cow G10); 1, 6, 4 (cow G9); 1, 6, 4 (cow B20) and 1, 1, 5 (cow G27).

Rumen contents from two more fistulated Friesian cows were used for in vitro studies on lipolysis. One (M21) in mid-lactation was treated similarly to the first four cows and received a final milk fat-depressing diet of 1, 2, 8 kg of hay, concentrates, and flaked maize, respectively, per day. The other cow (J), which was nonlactating, received an all-hay diet for 6 weeks and then a low-roughage diet of 20% hay and 80% flaked maize.

Sampling. Milk yields were recorded, and daily composite samples of the milk were analyzed for total fat content by the Gerber method (2) and for fatty acid composition by gas-liquid chromatography (26) with either a Perkin Elmer 880 or 900 model chromatograph. For characterizing the microflora, single samples of well mixed rumen digesta were taken from each of the lactating cows when given the high- and the low-roughage milk fat-depressing diets.

Characterization of the microflora. The Hungate (16) method of strict anaerobic culture as modified by Latham and Sharpe (17) was used throughout. All media for the enumeration of viable and lipolytic (tributyrin-hydrolyzing) bacteria and for the identification of isolates were based on medium 10 (4) as described by Latham, Sharpe and Sutton (18). Counts were made after 3 days of incubation, and the composition of the flora was determined by picking

and subsequently identifying all the colonies (100–150) from suitable roll tubes used for the enumeration of the total viable population.

Direct microscopic counts of formalized suspensions of protozoa in glycerol-water (1:1, v/v) were made in a Neubauer counting chamber (Hawksley & Sons Ltd., Lancing, England), and differential counts of bacteria were made by the Breed technique (1) by using Gram-stained preparations.

Lipolysis studies. Two methods were used for determining lipolytic activity.

(i) An agar diffusion method (19) was used to test the lipolytic activity of the bacteria from cow M21. The rate of hydrolysis of various triglycerides emulsified in buffered agar by test suspensions of bacterial cells was determined by measuring the diameter of the clear zones produced in the opaque gel at intervals during incubation at 30 C. Suitable suspensions of bacterial cells were obtained from 100 ml of rumen fluid which had been filtered through one layer of muslin. The filtered rumen fluid was centrifuged several times at approximately 300 × *g* for 5 to 10 min to remove the protozoa, and the supernatant fluid was centrifuged at 15,000 × *g* for 1 hr to sediment the bacteria. The bacteria were resuspended in 2.5 ml of 0.05 M sodium phosphate buffer, *p*H 7.0, and the amount of protein per milliliter of suspension was determined (20) so that in comparing the lipolytic activity between rations all values could be corrected to a unit value of 25 mg of protein per ml of suspension.

(ii) An incubation method was used to determine the lipolytic activity of whole rumen contents from cow M21 and of the protozoal, bacterial, and cell-free rumen fluid fractions of rumen contents from cow J when each cow was given high- and low-roughage diets. Sediments obtained by centrifuging at 300 × *g* (rich in protozoa but contaminated with feed particles and large numbers of bacteria) and 15,000 × *g* (rich in bacteria) were resuspended in their original volume of cell-free (15,000 × *g* supernatant fluid) rumen fluid. Duplicate flasks containing each of the rumen fluid fractions were incubated at 39 C in a water bath. Each flask was closed with a rubber bung adapted to allow continuous stirring and gassing with O_2-free nitrogen throughout the incubation. Emulsions of linseed oil or triolein were prepared by sonic oscillation (Dawe Soniprobe type 1130) in 0.05 M phosphate buffer, *p*H 7.0, containing 0.1% (w/v) gum acacia. The emulsion was purged with O_2-free nitrogen, and 10% (v/v, final incubation mixture) added to one (experimental) of each pair of flasks to give 10 mM final concentration of triglyceride. The same volume of phosphate buffer containing only gum acacia was added to the remaining flasks (controls). After thorough mixing, 20-ml subsamples were removed from both the experimental and control flasks (0 hr), the reaction was stopped by acidifying to *p*H 2.0 with 50% H_2SO_4, and the sample was shell frozen with CO_2-acetone. Additional subsamples were taken at 8 hr and treated similarly. All of the subsamples were then freeze-dried. Total lipid in the freeze-dried material was extracted in 2:1 (v/v) chloroform-methanol, and the nonesterified fatty acids in the extract were separated by thin-layer chromatography (28) and estimated quantitatively by the colorimetric method of Duncombe (10).

Hydrogenation studies. Samples of rumen digesta were taken 4 to 5 hr after feeding from animals on the control diet and again from the same animals after they were established on the low-roughage diet and showing depressed milk fat. These samples were taken directly into each of two sterile graduated flasks and immediately gassed with O_2-free nitrogen. Sufficient anaerobic dilution fluid (3) was added to dilute the contents by one-third to give a final volume of 1 liter. At the same time additional rumen samples were taken for microbiological analysis. The flasks were incubated in a similar manner to that used in the lipolysis studies. A 5-ml amount of 10% soybean oil emulsion ("Intralipid": Paines and Byrne, Greenford, England) was injected into the contents of one (experimental) of the flasks. After allowing 2 min for thorough mixing of the emulsion with the digesta, 30-g subsamples were taken from both the experimental and control flasks (0 hr), and again after 1, 2, 4, and 6 hr of incubation. The weight of each subsample was recorded, and the reaction was stopped by immediately transferring the sample into 2:1 (v/v) chloroform-methanol for extraction of total lipids. At each time of sampling, the *p*H and total numbers of bacteria were also recorded.

Extraction of total lipids and total fatty acids and determination of fatty acid composition. Total lipids from whole rumen contents in the hydrogenation studies and from freeze-dried rumen fluid fractions in the lipolysis studies were extracted in 2:1 (v/v) chloroform-methanol and washed with 0.88% (w/v) KCl solution as described by Folch, Lees, and Sloane Stanley (12). Total fatty acids were extracted from the total lipid samples after saponification, and their composition was determined by gas-liquid chromatography (26).

RESULTS

Milk fat. Although the composition of the low-roughage diets fed to individual animals differed, they all gave a marked depression in milk fat secretion. At the same time, the proportions of saturated fatty acids in the milk fat were decreased and the proportions of unsaturated fatty acids were increased (Table 1).

Rumen microflora. With the exception of cow G9, the low-roughage diets increased the number of viable bacteria in all cows by 0.6 to 1.6 log units but decreased the number of lipolytic (tributyrin-hydrolyzing) bacteria by up to 3 log units (Table 2). At the same time, these diets reduced by various amounts the numbers of ciliate protozoa. Of the two cows used in the lipolysis studies, M21 lost its ciliates, and there was a reduction in the number of ciliates in cow J which was not quantified. Only one (G10) of the four cows used in the hydrogenation studies lost its ciliates,

while in cow G27 they were reduced by 90%. However, the two remaining cows (G9 and B20) lost only 32 and 45% of their ciliates, respectively.

The variation in the composition of the bacterial flora between cows given the low-roughage diets was considerable (Table 3). The proportions of *Selenomonas*, *Peptostreptococcus*, *Bifidobacterium*, and *Lactobacillus* were particularly variable. Nevertheless, increases in lactogenic and propionogenic bacteria typical of low-roughage diets (4, 18) were observed. The most consistent effect of the low-roughage diets on the bacterial flora was the severe reduction in the proportion of *Butyrivibrio* spp. *Borrelia*, which like *Butyrivibrio* is capable of hydrogenation (23, 35), was also reduced.

Rumen lipolysis. Preparations of mixed rumen bacteria derived from cow M21 when fed the high-roughage diet hydrolyzed buffered emulsions of both tributyrin and tripalmitin, but tributyrin was hydrolyzed almost 2.5-fold as fast as tripalmitin (Fig. 1). On the low-roughage diet the rate of hydrolysis of both triglycerides by similar preparations of mixed rumen bacteria fell to one-fifth of the previously observed rates.

Incubations of whole rumen contents with emulsified linseed oil confirmed this apparent reduction in lipolytic activity (Table 4). Progressively less triglyceride was hydrolyzed as the roughage was reduced and the cereal content of the ration increased. In a separate experiment the lipolytic activity of the protozoal, bacterial, and cell-free rumen fluid fractions obtained from cow J given similar high- and low-roughage rations were compared. The bacterial fraction associated with the hay diet had the greatest lipolytic activity, hydrolyzing 87.6% of the added triglyceride (Table 5), whereas the protozoal and cell-free rumen fluid fractions hydrolyzed only 28.6 and 13%, respectively. The same fractions from animals on the low-roughage ration hydrolyzed 5.8, 34.9, and 5.7%, respectively, indicating a considerable decrease in lipolysis by the bacterial fraction offset to some extent by a slight rise in that of

TABLE 1. *Effect of high- and low-roughage diets on the mean content and composition of fat in the milk of four cows*

Component	Percentage of composition	
	High roughage	Low roughage
Milk fat	3.64 ± 0.24[a]	1.62 ± 0.21
Milk fatty acids[b]		
4:0	3.7 ± 0.39	2.7 ± 0.17
6:0	2.5 ± 0.18	1.1 ± 0.12
8:0	1.6 ± 0.10	0.4 ± 0.12
10:0	3.8 ± 0.17	1.7 ± 0.39
12:0	5.5 ± 0.35	3.9 ± 0.30
14:0	15.1 ± 0.35	10.9 ± 0.50
16:0	28.0 ± 1.99	25.7 ± 1.30
18:0	9.7 ± 0.92	4.6 ± 0.51
18:1	22.1 ± 2.00	35.8 ± 2.07
18:2	1.3 ± 0.25	4.7 ± 0.69

[a] ±Standard error of mean.

[b] Number of carbon atoms and number of double bonds (11).

TABLE 2. *Effect of high- and low-roughage milk fat-depressing diets on the counts of total viable and lipolytic bacteria and total ciliate protozoa in five lactating cows*

Cow	High roughage			Low roughage		
	Bacteria		Ciliate protozoa × 10^{-4}/g	Bacteria		Ciliate protozoa × 10^{-4}/g
	Total viable × 10^{-9}/g	Lipolytic × 10^{-6}/g		Total viable × 10^{-9}/g	Lipolytic × 10^{-6}/g	
G9	2.8	5.0	11.0	2.8	2.4	7.5
G10	0.6	4.5	21.0	24.0	0.2	None
B20	0.4	49.0	8.0	25.0	>0.1	4.4
G27	5.9	19.0	11.0	27.0	0.3	0.9
M21	2.0	24.0	13.0	12.0	>0.02	None

TABLE 3. *Effect of high- and low-roughage milk fat-depressing diets on the bacterial flora of five lactating cows*

Organism	Composition (mean % total isolates)	
	High roughage	Low roughage
Butyrivibrio fibrisolvens	25.0 ± 4.0[a]	2.3 ± 1.8
Bacteroides spp.	11.3 ± 2.8	3.7 ± 0.5
Selenomonas spp.	6.9 ± 1.3	19.3 ± 15.1
Ruminococcus spp.	11.1 ± 2.3	9.6 ± 1.6
Peptostreptococcus elsdenii	5.8 ± 2.1	14.4 ± 4.7
Streptococcus bovis	4.0 ± 1.2	8.0 ± 0.3
Lactobacillus spp. and *Bifidobacterium* spp.	5.7 ± 1.8	20.3 ± 6.3
Lachnospira multiparus	0.4 ± 0.4	1.9 ± 0.8
Succinivibrio dextrinosolvens	1.4 ± 0.6	0.6 ± 0.5
Eubacterium ruminantium	2.0 ± 0.9	0.6 ± 0.5
Borrelia spp.	0.4 ± 0.4	None
Succinimonas amylolytica	0.6 ± 0.6	None
Unidentified isolates		
Gram-negative rods	8.7 ± 2.0	6.8 ± 2.8
Gram-variable cocci	1.0 ± 0.6	7.2 ± 4.1
Others	15.7	5.3

[a] ±Standard error of mean.

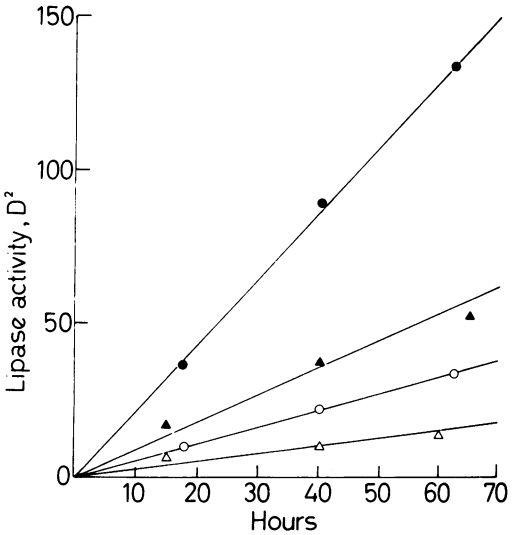

FIG. 1. *Changes in the rate of hydrolysis of tributyrin and tripalmitin by suspensions of mixed bacteria from cow M21 when fed high- and low-roughage diets. Tributyrin, high roughage (●), low roughage (○). Tripalmitin, high roughage (▲), low roughage (△). Each point is the mean of four separate experiments. Hydrolysis was determined by method of Lawrence et al. (19) where D^2 = square of the difference between the diameter of the zone of hydrolysis and the diameter of the well in millimeters.*

the protozoal fraction. In both experiments the level of unesterified fatty acid of dietary origin increased with increases in the proportion of concentrates in the diet.

Rumen hydrogenation. When the cows were fed the low-roughage diets, the concentrations of total lipid and fatty acids in the rumen digesta were two or three times greater than when they were fed the high-roughage diets. These differences can be accounted for by higher dietary intakes of lipid on the low-roughage rations and by increases in the contribution of rumen microbial lipid (29). Although there were appreciable amounts of oleic and linoleic acids of dietary origin present in the in vitro incubation systems, it has been assumed that any differences in the composition of the total C_{18} fatty acids between samples from the control and experimental incubations reflected metabolism of the added soybean oil.

The patterns of hydrogenation obtained were similar for all four cows examined, and the mean results for the high- and low-roughage diets are shown in Fig. 2 and 3, respectively. Over the 6-hr incubation period, the hydrogenation of linolenic and linoleic acids added in the form of soybean oil was, on the low-roughage diet, 59 and 63%, respectively, of that obtained on the high-roughage ration. The overall reduced rates of hydrogenation on the low-roughage diet were significant at the 5% and 1% level for linolenic and linoleic acids, respectively. The pattern of hydrogenation also differed between the two diets; with the high-roughage diet the hydrogenation appeared to commence soon after addition of the soybean oil and to progress rapidly up to the end of the fourth hour of incubation, whereas with the low-roughage ration the most rapid hydrogenation occurred between the second and sixth hours.

DISCUSSION

The decrease in milk fat secretion and changes in milk fat composition which occurred with the low-roughage diets fully support observations from earlier experiments with similar diets (25, 26). The increased proportion of unsaturated fatty acids in the milk fat on the low-roughage diet was probably a reflection of both increased intake of dietary unsaturated acids and a decrease in their rumen hydrogenation. The latter effect was confirmed by the results obtained from the in vitro incubations of rumen digesta with emulsified soybean oil.

Changes in the composition or physical form of a diet are well known to affect the composition of the rumen microflora. In a previous study (18), the composition of the rumen bacterial flora of nonlactating cows fed both high- and low-roughage diets did not vary greatly between cows fed the same ration, but in the present experiment the between-cow variation on the low-roughage diet was large and was extended to the protozoa. The reason for this variability was not clear but may have been related to the high food intake of the lactating cows.

It has been shown that, when emulsions of fats or oils are added to rumen digesta, they are rapidly adsorbed on food particles and microorganisms (13, 14); Czerkawski (8) demonstrated that levels of linseed oil in excess of 300 mg/120 ml of rumen fluid inhibited hydrogenation. Greater concentrations of triglycerides than this, while saturating the lipolytic activity of rumen fluid (Demeyer, *personal communication*), have not been shown to inhibit lipolysis. In the present experiment, the concentration of total lipid and fatty acid in the rumen digesta used for the in vitro determinations of the lipolytic and hydrogenating activities was greater with the low-roughage than with the high-roughage diets, and, while in the hydrogenation studies the substrate triglyceride

TABLE 4. *Effect of high- and low-roughage diets on the in vitro hydrolysis of a 10 mM emulsion of linseed oil by whole rumen contents from cow M21*

Ration	Flask	Unesterified fatty acid (μmoles/100-ml incubation mixture)		Percent triglyceride hydrolyzed
		Produced during 8-hr incubation	Produced from hydrolysis of linseed oil (by difference)	
High roughage	Control	140		
	Plus linseed oil	2,686	2,546	84.9
Intermediate low roughage	Control	330		
	Plus linseed oil	1,506	1,176	39.2
Low roughage (milk fat depressing)	Control	770		
	Plus linseed oil	1,310	540	18.0

TABLE 5. *Effect of high- and low-roughage diets on the in vitro hydrolysis of a 10 mM emulsion of triolein by various fractions of rumen contents obtained from cow J*

Fraction	Flask	Unesterified fatty acid (μmoles/100-ml incubation mixture)		Percent triglyceride hydrolyzed
		Produced during 8-hr incubation	Produced from hydrolysis of triolein (by difference)	
High roughage				
Bacterial	Control	15		
	Plus triolein	2,641	2,626	87.6
Particulate	Control	72		
	Plus triolein	932	860	28.6
Cell-free fluid	Control	9		
	Plus triolein	399	390	13.0
Low roughage				
Bacterial	Control	75		
	Plus triolein	227	152	5.8
Particulate	Control	281		
	Plus triolein	1,329	1,048	34.9
Cell-free fluid	Control	35		
	Plus triolein	205	170	5.7

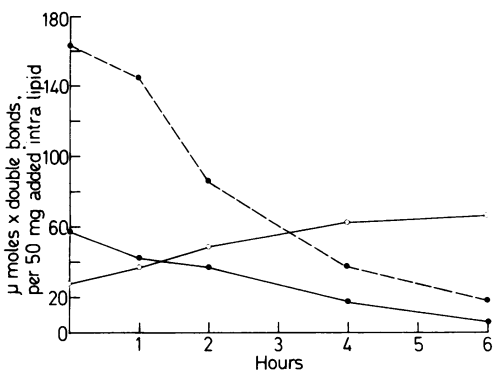

FIG. 2. *Changes in the content of linolenic (●——●), linoleic (●-----●), and oleic (O——O) acids in "Intralipid" during incubation with rumen contents from cows fed a high-roughage diet.*

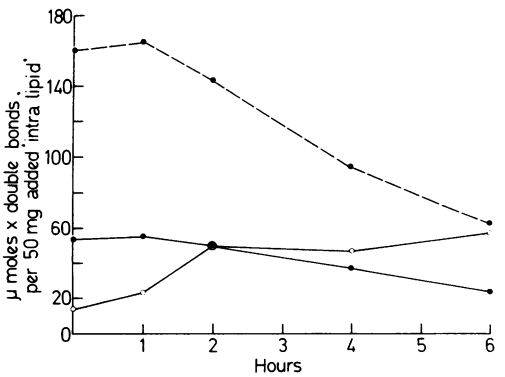

FIG. 3. *Changes in the content of linolenic (●——●), linoleic (●-----●), and oleic (O——O) acids in "Intralipid" during incubation with rumen contents from cows fed a low-roughage diet.*

was added at the rate of 50 mg/100 ml of digesta, more than 800 mg/100 ml of digesta was added in the lipolysis studies. However, serial samples taken during the various incubation experiments revealed no major change in either the direct count or motility of bacteria and protozoa, indicating that even at the high substrate levels used in the lipolysis studies the added triglyceride had no apparent deleterious effect on the microflora.

It is probable that the reduced lipolytic activity of the rumen digesta found with the low-roughage diets was related to the reduced numbers of lipolytic vibrios. These diets greatly reduced the viable counts of tributyrin-hydrolyzing bacteria, and approximately 70% of the tributyrinolytic bacteria isolated on a previous occasion in this laboratory from the same medium were found to resemble butyrivibrios. Five out of 25 of these strains were able to hydrolyze long-chain triglycerides up to triolein. In the present work, the strongly lipolytic vibrio *Anaerovibrio lipolytica* was not isolated, and subsequent work in this laboratory suggests that this and similar lipolytic organisms constitute no more than 1% of the viable population. This effect of diet on lipolysis is in agreement with recent observations of Demeyer (*personal communication*) who found that the inclusion of sucrose in the diet of a sheep or of glucose in an in vitro incubation of rumen fluid reduced lipolysis.

It is not clear why the lipolytic activity of the protozoal fraction should have increased with the low-roughage diet. Clarke and Hawke (7) found that much of the lipolytic activity of this fraction could be transferred to the bacterial fraction after homogenization, and they therefore concluded that many lipolytic bacteria were attached to the particulate matter. Since on the low-roughage diet many starch granules will also sediment at the low "*g*" values used for separating the protozoal fraction of rumen fluid, they will, in so doing, bring with them even larger numbers of adhering bacteria among which may be actively lipolytic strains.

As indicated by the changes in milk fat composition and from the results of the in vitro incubations, the hydrogenating activity of the rumen contents was also reduced by the low-roughage rations. Strains of *Butyrivibrio fibrisolvens* were the first rumen bacteria to be implicated in the biohydrogenation of polyunsaturated fatty acids and were found to hydrogenate to the monoene (23). Since then laboratory strains of *Borrelia* (35), a gram-negative micrococcus (22), a strain each of *Eubacterium* and *Ruminococcus* (34), and two strains of cellulolytic *Clostridium* spp. (32) have all been found to biohydrogenate in a similar manner. However, only two strains of bacteria have so far been reported to be capable in pure culture of the complete reductive process to the saturated acid (34). Under normal conditions of high-roughage feeding, *Butyrivibrio fibrisolvens* is by far the most numerous of all of these biohydrogenating organisms found in the rumen and in the present experiment comprised 25.0% of the viable population in cows fed the high-roughage diets. On the assumption that the isolates of *Butyrivibrio* and *Borrelia* included hydrogenating strains, the reduction in their numbers which occurred with the low-roughage diets is consistent with the decrease in hydrogenation observed in vitro. Tove and Matrone (30) reported that, compared with conventional diets, the reduction of the monoenoic to the saturated acid proceeded more slowly with high-concentrate diets. As most workers have not been able to isolate strains which can carry out this final reductive step, such organisms are unlikely to occur in the majority flora, yet, like the butyrivibrios, they would appear to be greatly affected by dietary changes.

Although the protozoa-rich fraction of rumen contents has been shown to have biohydrogenating activity (5), the role of individual species of ciliate protozoa in this activity remains equivocal (31). However, studies on the plasma lipids of lambs with or without rumen ciliates indicate that dietary C_{18} unsaturated fatty acids are more effectively hydrogenated in the presence of ciliates (A. K. Lough, Proc. Nutr. Soc., **27**:30A, 1968). It is therefore of interest that in the present work the reduction in hydrogenation induced by the low-roughage diets was no greater in the two cows which either lost or suffered a 90% reduction in the numbers of ciliates than in the remaining cows which retained between 55 and 70% of their ciliates. Because Hawke and Silcock (15) have recently shown that only nonesterified fatty acids can be hydrogenated, the present results suggest that, in addition to the adverse effects of low-roughage rations on the numbers of potential biohydrogenating organisms, a reduction in lipolysis may also contribute to the observed reduction in hydrogenation.

ACKNOWLEDGMENTS

The analysis of total fatty acid composition by A. J. Hall, the care of the animals by V. W. Johnson, and the capable technical assistance of Ann Siddell are gratefully acknowledged.

LITERATURE CITED

1. British Standard Institution. 1968. Method of microbiological examination for dairy purposes. B.S. 4285.
2. British Standards Institution. 1969. Gerber method for the determination of fat in milk and milk products. B.S. 696, part 2.
3. Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205-217.
4. Caldwell, D. R., and M. P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14**:794-801.
5. Chalupa, W., and A. J. Kutches. 1968. Biohydrogenation of linoleic $1^{14}C$ acid by rumen protozoa. J. Anim. Sci. **27**:1502-1508.
6. Chalupa, W., G. D. Odell, A. J. Kutches, and R. Lauker. 1967. Changes in rumen chemical characteristics and protozoa populations of animals with depressed milk fat tests. J. Dairy Sci. **50**:1002, P154.
7. Clarke, D. G., and J. C. Hawke. 1970. Studies on rumen metabolism. VI. In vitro hydrolysis of triglyceride and isolation of a lipolytic fraction. J. Sci. Food Agric. **21**:446-452.
8. Czerkawski, J. W. 1967. Incubation inside the bovine rumen. Brit. J. Nutr. **21**:865-878.
9. Davis, C. L., and D. S. Sachan. 1966. Effect of feeding and milk fat depressing ration on fatty acid composition of blood lipids. J. Dairy Sci. **49**:1567-1569.
10. Duncombe, W. G. 1963. The colorimetric micro-determination of long chain fatty acids. Biochem. J. **88**:7-10.
11. Farquhar, J. W., W. Insull, Jr., P. Rosen, N. Stoffel, and E. H. Ahrens, Jr. 1959. The analysis of fatty acid mixtures by gas-liquid chromatography. Nutr. Rev. **17**:(8) part II, suppl.
12. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**:497-509.
13. Garton, G. A. 1967. The digestion and absorption of lipids in ruminant animals. World Rev. Nutr. Diet. **7**:225-250.
14. Hawke, J. C., and J. A. Robertson. 1964. Studies on rumen metabolism. II. In vivo hydrolysis and hydrogenation of lipid. J. Sci. Food Agric. **5**:283-289.
15. Hawke, J. C., and W. R. Silcock. 1970. The in vitro rates of lipolysis and biohydrogenation in rumen contents. Biochim. Biophys. Acta **218**:201-212.
16. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1-49.
17. Latham, M. J., and M. E. Sharpe. 1971. The isolation of anaerobic organisms from the bovine rumen, p. 133-147. *In* D. A. Shapton and R. G. Board (ed.), Isolation of anaerobes. Academic Press Inc., London.
18. Latham, M. J., M. E. Sharpe, and J. D. Sutton. 1971. The microbial flora of the rumen of cows fed hay and high cereal rations and its relationship to the rumen fermentation. J. Appl. Bacteriol. **34**:425-434.
19. Lawrence, R. C., T. F. Fryer, and B. Reiter. 1967. Rapid method for the quantitative estimation of microbial lipase. Nature (London) **213**:1264-1265.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
21. McCarthy, R. D., P. S. Dimick, and S. Patton. 1966. Field observations on lipids of cows with depressed milk fat. J. Dairy Sci. **49**:205-208.
22. Mills, S. C., T. W. Scott, G. R. Russell, and R. M. Smith. 1970. Hydrogenation of C_{18} unsaturated fatty acids by pure cultures of a rumen micrococcus. Aust. J. Biol. Sci. **23**:1109-1113.
23. Polan, C. E., J. J. McNeill, and S. B. Tove. 1964. Biohydrogenation of unsaturated fatty acids by rumen bacteria. J. Bacteriol. **88**:1056-1064.
24. Slyter, L. L., R. R. Oltjen, D. L. Kern, and F. C. Blank. 1970. Influence of type and level of grain and diethylstilbestrol on the rumen microbial populations of steers fed all-concentrate diets. J. Anim. Sci. **31**:996-1002.
25. Storry, J. E., and J. A. F. Rook. 1966. The relationship in the cow between milk-fat secretion and ruminal volatile fatty acids. Brit. J. Nutr. **20**:217-228.
26. Storry, J. E., J. A. F. Rook, and A. J. Hall. 1967. The effect of the amount and type of dietary fat on milk fat secretion in the cow. Brit. J. Nutr. **21**:425-438.
27. Storry, J. E., and J. D. Sutton. 1969. The effect of change from low roughage diets on rumen fermentation, blood composition and milk fat secretion in the cow. Brit. J. Nutr. **23**:511-521.
28. Storry, J. E., and B. Tuckley. 1967. Thin-layer chromatography of plasma lipids by single development. Lipids **2**:501-502.
29. Sutton, J. D., J. E. Storry, and J. W. G. Nicholson. 1970. The digestion of fatty acids in the stomach and intestines of sheep given widely different rations. J. Dairy Res. **37**:97-105.
30. Tove, S. B., and G. Matrone. 1962. Effect of purified diets on the fatty acid composition of sheep tallow. J. Nutr. **76**:271-277.
31. Viviani, R. 1970. Metabolism of long-chain fatty acids in the rumen. Advan. Lipid Res. **8**:267-346.
32. Viviani, R., A. R. Borgatti, and D. Matteuzzi. 1968. Isolation of typical rumen bacteria active in unsaturated fatty acid biohydrogenation. Boll. Soc. Ital. Biol. Sper. **44**:2185-2189.
33. Viviani, R., A. R. Borgatti, P. G. Monetti, and A. Mordenti. 1967. Studies on long chain fatty acids composition in rumen fluid during the development of ruminal function in the calf. Zentralbl. Veterinaermed. Reihe A **14**:833-844.
34. White, R. W., and P. Kemp. 1971. Sheep rumen bacterial isolates which biohydrogenate unsaturated dietary fatty acids. J. Gen. Microbiol. **68**:vi.
35. Yokoyama, M. T., and C. L. Davis. 1971. Hydrogenation of unsaturated fatty acids by *Treponema* (*Borrelia*) strain $B_{s}5$, a rumen spirochete. J. Bacteriol. **107**:519-527.