The Magnitude of the Microbial Fermentation in the Bovine Rumen[1]

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The kinds and amounts of volatile acids absorbed from the rumen of sheep have been estimated by comparing the volatile acids in the blood draining the rumen with the amount in the peripheral circulation (Barcroft *et al.*, 1944; Kiddle *et al.*, 1951). An undesirable factor in these experiments is the disturbance of normal conditions by the operative procedures (Masson and Phillipson, 1951). Also, some of the acids are metabolized by the tissues of the rumen wall (Pennington, 1952) and the total amount leaving the rumen does not appear in the blood. These drawbacks have been overcome (Danielli *et al.*, 1945; Pfander and Phillipson, 1953) in experiments with fistulated sheep by determining the rate of disappearance of the various volatile acids from the rumen rather than their appearance in the blood.

Another approach to the problem, one particularly applicable to the bovine animal, is to determine the rate at which the acids are formed. This was used by Stone (1949) to compare microbial activities in the normal and atonic rumen. The volatile acids produced in the rumen are completely utilized by the bovine animal since only negligible amounts are excreted (Wilsing, 1885; Elsden *et al.*, 1946). Rates of production of volatile acid cannot be determined directly in the rumen because absorption decreases the acid concentration simultaneously with its increase through fermentation. Rates of production can be estimated indirectly if absorption is eliminated by removing the rumen contents from the animal.

Removal of rumen contents is subject to the limitations 1) that it may immediately impose unnatural conditions on the microbiota, and 2) even if no unnatural conditions are immediately imposed, the microbial activities, dissociated from the absorptive and secretory activities of the host, soon lead to abnormal conditions.

Experience in culturing cellulolytic bacteria (Hungate, 1950) and protozoa (Hungate, 1942) of the rumen has indicated that anaerobiosis, proper temperature, and maintenance of suitable pH and a high buffering capacity (carbon dioxide-bicarbonate buffer) are important. If these factors are properly regulated, removal of rumen contents need not immediately modify the normal microbial activities.

The second limitation, that fermentation by rumen microorganisms *in vitro* leads to abnormal conditions, can be avoided if the rate of increase in the volatile acids is determined at the instant rumen contents are removed. This is experimentally impossible because rates of production must be calculated from the difference in volatile acid content at zero and some subsequent finite time. However, the volatile acid concentration can be determined in a portion of the sample at the instant of removal from the rumen and, by shortening as much as possible the incubation period before the next portion is analyzed, an experimentally determined average production rate for the short interval can be calculated. From analyses made after further incubation a curve of volatile acid content against time can be constructed, the slope of which, for any particular time, represents the rate of production of volatile acid. The slope at zero time is the rate of volatile acid production at the instant of removal from the rumen. This rate should be identical with that of the contents remaining in the rumen.

Methods

Rumen contents were removed from a fistula animal[2] to a flask previously filled with carbon dioxide with a gentle stream of carbon dioxide flowing through it from a capillary tube. A sample for analysis was immediately (zero hour) acidified with 25 milliequivalents of sulfuric acid per 40 g of rumen contents to stop all metabolic processes. The remaining rumen contents were protected from exposure to oxygen by stoppering the flask as the capillary tube was withdrawn. The flask and contents were taken to the laboratory as soon as possible and incubated in a water bath at 39 C. If the weather was cold the flask was kept in a bucket of water at 39 C during the time it was in transport to the laboratory.

Additional samples were removed from the flask at 1, 2, 3 and 4 hours, and acidified, care always being taken to avoid exposure to oxygen. They were diluted

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[2] Fistula animals were supplied through the cooperation of the College of Veterinary Medicine and the Department of Animal Husbandry.

TABLE 1. *Recovery of volatile acids*

	RUN 1	RUN 2	RUN 3	RUN 4	RUN 5
Acetic acid.....	0.0966	0.0948	0.0950	0.0961	0.0956
Propionic acid...	0.0374	0.0356	0.0346	0.0352	0.0357
Butyric acid.....	0.0164	0.0168	0.0168	0.0177	0.0169
Total.....	0.1504	0.1472	0.1464	0.1490	0.1482

with an equal volume of water and allowed to stand at room temperature for at least 24 hours with occasional shaking to insure equilibrium between the acid in the liquid and solid phases. After settling, aliquots of the liquid were removed and steam distilled for volatile acids. The distillate was titrated with barium hydroxide in a carbon dioxide-free atmosphere using brom thymol blue indicator, and the salts were evaporated to dryness on a steam bath. The error involved in the steam distillation was relatively small, duplicate aliquots usually differing by less than 1 per cent, though in the grain experiments a greater error was involved.

The individual volatile acids were separated chromatographically according to a modification of the method of Elsden (1946). The barium salts were transferred with a few ml of water to a tube 100 x 20 mm diameter and the water again evaporated off. The residual salts were dissolved in 0.2 ml of hot water and the tube then placed in an ice bath. Four ml of 5 per cent butanol in chloroform (CB_5) were added and then 1 g of $K_2S_2O_7$. These were thoroughly mixed until the indicator no longer showed any blue color, then 2 more g of $K_2S_2O_7$ were added and mixed. After standing a few minutes, the mixture was transferred to a sintered glass filter and the CB_5 forced through with pressure from carbon dioxide-free air. Additional portions (3 to 4 ml) of CB_5 were used for further extraction until the volume of filtrate was about 15 ml. Then CB_{20} was used for extraction until the volume of filtrate was 20 ml. Three-ml aliquots of this solution of volatile acids in chloroform-butanol, containing usually 0.08 to 0.15 mEq acid, were run on the chromatographic column.

The column was prepared by thoroughly mixing 6 g of cellulose[3] with 2.7 ml of brom cresol green indicator solution and 0.1 ml of 0.2 normal NaOH. The cellulose flour had previously been thoroughly washed in distilled water and dried. The mixture was suspended in 90 ml of CB_1 and transferred in 10-ml quantities to a glass tube 45 cm long and 16 mm in diameter. A screen with a little absorbent cotton over it formed the support at the bottom of the column. After each addition of the suspension, the cellulose was firmly packed with a plunger constructed by soldering a porous flat disc onto the end of a long rod and tying a linen cloth over the end. The final column was about 15 cm long.

The sample containing the volatile acids was added gently at the top of the column with a pipette, avoiding contact with the upper walls of the tube. After the solvent entered the column, one or two applications of 1 ml of chloroform were used to wash in all of the acids. The column was developed with pure chloroform until the butyric band had been extracted, then CB_1 was used to obtain the propionic acid. Acetic acid was removed by using CB_5.

The acid from each band was titrated with barium hydroxide in a carbon dioxide-free atmosphere of nitrogen. Acids were identified initially by their Duclaux distillation constants, later by their Rf values.

The accuracy of the chromatographic separation was tested by preparing a mixture containing 0.096 milliequivalents of acetic acid, 0.0361 mEq of propionic acid, and 0.0175 mEq of butyric acid, a total of 0.1496 mEq. The acids were neutralized, dried, extracted with chloroform-butanol, and run on the chromatograph. The amounts of each acid recovered are shown in table 1.

EXPERIMENTAL RESULTS

A. Total Volatile Acid

The first two experiments were designed to compare the rates of production of volatile acids by rumen liquid as compared with the total rumen contents which contain a higher proportion of solids (about 14 per cent solids in the hay-fed animal). The amounts of volatile acid at various times in samples of rumen liquid and of total rumen contents from fistula steer number 1 are shown in table 2. The material for both experiments was collected at 10:20.

In experiment 1 the total rumen contents showed a higher volatile acid content at zero time than did the liquid. This may be explained by a lack of equilibrium between liquid and solid phases in the rumen. If acids are formed primarily in the solids they would be expected on occasion to be present in greater amounts than in the liquid. Addition of saliva and absorption of acids would tend to maintain such a gradient between solids and liquid. The reverse of this relationship in experiment 2 may have been due to recent ingestion of hay. In spite of the difference in the direction of the initial gradient of volatile acid concentration between liquid and solids in the two experiments, both agree in showing a much greater increase of volatile acids in the sample containing solids. This is presumably due to the greater amount of available substrate. The average volatile acid increases are plotted in figure 1.

The necessity of using total rumen contents with their contained solids greatly increased the difficulty in obtaining representative samples. No technique for

[3] BW-200 Solka-Floc, Brown Co., Berlin, N. H.

TABLE 2. *Comparison of volatile acid in rumen liquid with that in liquid plus solids at various times after removal*

EXPERIMENT NO.	0 HR		1 HR		2 HR		3 HR		4 HR	
	Liquid	Liquid plus solids	Liquid	Liquid plus solids	Liquid	Liquid plus solids	Liquid	Liquid plus solids	Liquid	Liquid plus solids
1	13.28*	16.08	13.54	17.38	13.85	18.14	13.89	18.58	13.93	18.75
2	9.35	8.82	9.47	9.90	9.49	10.80	9.55	11.50	9.66	12.07
Av.	11.32	12.45	11.51	13.64	11.67	14.47	11.72	15.04	11.80	15.41

* Milliequivalents per 100 g.

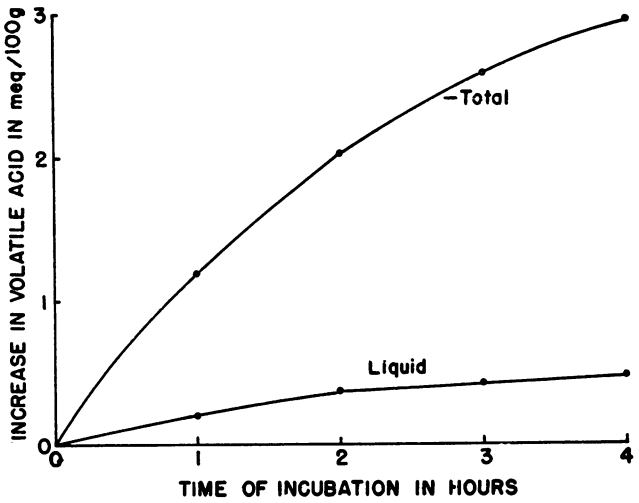

FIG. 1. Volatile acid in incubated rumen liquid and in total contents.

reliable sampling was discovered, though use of large samples (ca. 500 g) reduced the error from this source.

The effect on volatile acid production of diluting the rumen contents with a balanced salt solution (Hungate, 1950) was studied in experiment 3. The procedure was similar to that previously used except that before incubation one sample of contents was mixed with an equal volume of the salt solution which had been buffered, made anaerobic, and adjusted to a temperature of 39 C.

Material for this experiment was obtained at 10:00 by stomach tube from fistula animal number 2 which had been on a green grass pasturage diet. The amounts of volatile acids present at various times are shown in table 3.

These results do not indicate any significant difference in the rate of production of volatile acid in the two samples. The initial pH of 6.45 dropped after 4 hours to a value of 5.6 in the diluted culture and 5.5 in the undiluted. Although the total amounts of volatile acids in diluted and undiluted contents were the same, there was a possibility that the acids differed in composition. This was tested by determining the amounts of the individual acids present at 1 hour, with results as shown in table 4.

This experiment is cited in detail to show the degree of reproducibility with the chromatographic separation

TABLE 3. *Comparison of the amount of volatile acid in incubated diluted and undiluted rumen contents*

	0 HR	1 HR	2 HR	3 HR	4 HR
Diluted	7.12*	8.05	8.99	9.82	10.45
Undiluted	7.12	8.11	8.97	9.86	10.59

* Milliequivalents per 100 ml.

TABLE 4. *Composition of the volatile acid in diluted and undiluted rumen contents as estimated in chromatographic separations*

	BUTYRIC ACID	PROPIONIC ACID	ACETIC ACID
	*mEq**	*mEq*	*mEq*
Zero hour			
1st column	0.0175	0.0275	0.0917
2nd "	0.0173	0.0297	0.0884
3rd "	0.0162	0.0266	0.0932
Average	0.0170	0.0279	0.0911
1 hr, undiluted			
1st column	0.0155	0.0262	0.0841
2nd "	0.0162	0.0264	0.0824
3rd "	0.0142	0.0260	0.0846
Average	0.0153	0.0262	0.0837
1 hr, diluted			
1st column	0.0153	0.0261	0.0833
2nd "	0.0151	0.0235	0.0833
3rd "	0.0162	0.0278	0.0810
4th "	0.0158	0.0266	0.0824
Average	0.0156	0.0260	0.0824

* Actual titration values for individual acids extracted from each column.

in an actual experiment. The averages in table 4 have been recalculated to give the amount of each volatile acid per 100 g of rumen contents.

The slightly greater amount of the individual volatile acids in the diluted rumen contents (table 5) is due to their greater recovery in the chloroform-butanol extract preparatory to chromatographic separation. The percentage composition of the recovered acid in the diluted and undiluted sample is almost identical. In view of the similarity in total volatile acid, it must be concluded that during the first

TABLE 5. *Amounts of individual volatile acids per 100 g rumen contents in diluted and undiluted samples*

	BUTYRIC ACID 1 HR		PROPIONIC ACID 1 HR		ACETIC ACID 1 HR		RECOVERY OF VOLATILE ACID 1 HR
	mEq	*per cent*	*mEq*	*per cent*	*mEq*	*per cent*	*per cent*
Undiluted....	0.95	12.2	1.63	21.0	5.20	66.8	96
Diluted.....	1.01	12.6	1.68	21.0	5.33	66.4	100

TABLE 6. *Volatile acid in rumen contents removed at various times*

EXPERIMENT NO., DATE, HOUR, AND REMARKS	TIME AFTER FEEDING	TIME AFTER REMOVAL OF CONTENTS				
		0 hr	1 hr	2 hr	3 hr	4 hr
	hr	*mEq/100 g*				
Oct. 30						
4. 8:10, animal eating	0	9.33	11.86	14.30	14.26	15.16
5. 10:05	2	11.51	12.97	13.78	15.20	15.84
6. 14:05, just drank	6	14.74	16.37	17.15	18.00	18.54
7. 20:05, little liquid in rumen	2	17.09	17.85	19.40	20.01	22.05
Nov. 9						
8. 7:15	13	10.48	12.04	13.10	13.99	15.45
9. 9:55	16	8.57	9.52	10.19	11.85	11.90
10. 15:15	2	13.80	15.60	17.48	17.86	17.95
11. 20:00	7	18.58	19.80	21.59	21.56	21.09
Oct. 11						
12. 9:45		11.47	12.26	13.10	14.47	15.57

hour any alteration in the fermentation due to dilution with salt solution was too small to be detected.

In estimating the total volatile acid available to the ruminant, the rate of production at different times of day must be taken into consideration. The importance of this was examined by removing rumen contents at different times during the same day and following the volatile acid content of each sample. The procedure was repeated 10 days later using the same animal, fistula steer number 1. It was kept on a hay diet and fed regularly at 8:00 and 18:00 hours until the day of the second experiment. On the day of the second run it received only a double portion of feed at 13:00. The results are shown in table 6. Also shown in table 6 are the results for one additional experiment, steer number 12, with a hay diet.

The time since the last feeding was considered to be the most important single factor affecting the rate of fermentation. The experiments were grouped roughly according to this criterion and the results for experiments 5, 7 and 10 were averaged to give the production rate 2 hours after feeding. Similarly, experiments 6 and 11 were averaged to give the value at 6.5 hours and experiments 8 and 9 to give the value 14.5 hours after feeding. The results are plotted in figure 2.

Although the experimental error in individual analyses is fairly large, the increases in volatile acid shown in figure 2 do not indicate great differences in

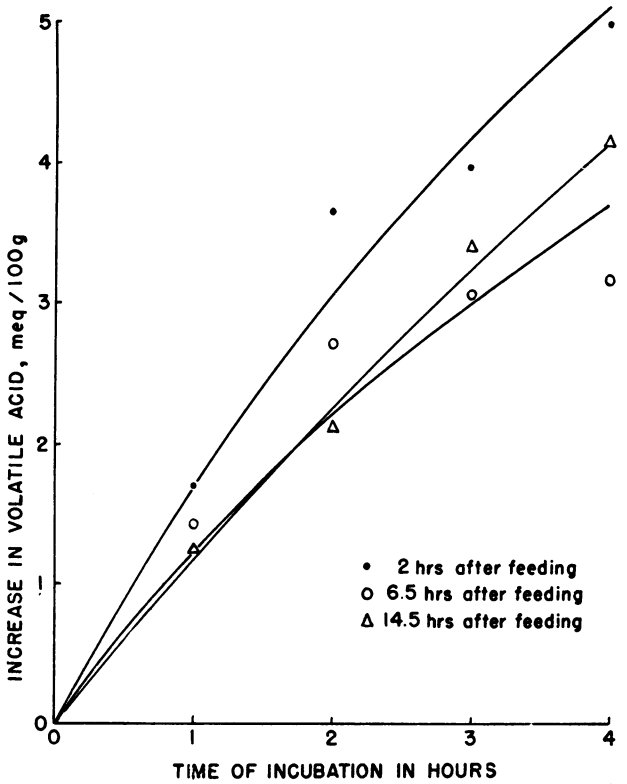

FIG. 2. Volatile acid in rumen contents incubated at different times after feeding.

volatile acid production at different times of day. Even 14.5 hours after the last feeding the rate of volatile acid production per 100 g rumen contents is almost as great as at 2 hours.

In table 6 the volatile acid concentration in rumen contents (zero hour) is seen to increase during the day. The low value at 9:55 was due to postponement of the morning feeding. Because, under normal feeding conditions, an increase in volatile acid concentration in the rumen during the day might be significant in evaluating rates of volatile acid production, some additional analyses for volatile acids in the rumen at various times were made. For one animal the volatile acids in both liquid and total contents were determined, in the other only the liquid was used. The results are collected in table 7.

The tendency for the volatile acid concentration in the rumen to increase during the day is not as apparent in the data of table 7 as in the experiments reported in table 6. Fistula animal number 2 had feed available at all times and the diurnal variation in volatile acid

TABLE 7. *Volatile acid in rumen contents at different times of day*

FISTULA ANIMAL NO.	MATERIAL USED	DEC. 3 23:30	DEC. 4 7:40	DEC. 4 10:00	DEC. 4 13:20	DEC. 4 17:30	DEC. 4 22:10
		mEq/100 g					
1	Total contents	11.83	13.95	14.88	16.88	15.48	14.20
1	Liquid	9.75	13.47	13.77	14.04	11.84	11.23
2	Liquid	11.44		9.52	8.03	10.01	9.30

content was less than with the first animal when it was fed at 8:00 and 18:00. Taking all the data into account, the volatile acid content during the day is somewhat higher than at night, suggesting that the rate of production exceeds the rate of absorption. Absorption apparently exceeds the rate of production during the night.

Rumen contents for three additional experiments were removed by stomach tube from a young unfistulated bull on a diet of two-thirds grain and one-third hay. These samples contained more liquid (dry matter about 5 per cent) than the rumen contents obtained through the fistula of the hay-fed animals, but appeared to be quite uniform. Later, one run was made on this animal during the time it was on grass pasturage. One run (in addition to the run reported in table 2) was made with the second fistula steer when it was on similar pasturage. The dry matter in the rumen contents of the animals on pasture was also about 5 per cent. All grain and pasturage experiments were performed with samples obtained by stomach tube. The results of these additional analyses are shown in table 8.

The curves obtained by averaging the values for the 11 experiments with hay, the 3 experiments with grain, and the 3 experiments, (experiments 3, 16 and 17) with grass pasturage, respectively, are shown in figure 3.

Inspection of figure 3 shows that the amount of volatile acid produced in the first hour on the hay diet was less than the amount produced from the grain diet, but greater than the amount from pasturage. The shape of the curve is that which would be expected for *in vitro* rumen contents. Fermentation slows down as products accumulate and the substrate diminishes.

The average concentration of volatile acid in rumen contents at zero hour was 12.77 mEq per 100 g for the hay experiments, 9.15 for the grain, and 8.30 for the pasturage experiments. The average increase of acid in rumen contents of hay-fed animals during the first hour of incubation was 1.37 mEq/100 g with a standard error of 0.15. The average increase in acid during the first hour with the grain diet was 2.01 mEq/100 g with a standard error of 0.10. The average increase during the first hour with the pasturage diet was 0.91 mEq/100 g with a standard error of 0.05.

The experimentally determined increases in volatile acid during the first hour of incubation are presumably less than the rates of production in the rumen because

TABLE 8. *Results of experiments with animals on pasturage or high grain diets*

EXPERIMENT NO.	TIME SAMPLE COLLECTED	TIME FED	0 HR	1 HR	2 HR	3 HR	4 HR
High grain diet—unfistulated bull							
			mEq/100 g				
13	15:40	12:40	10.36	12.48	13.85	14.56	15.18*
14	9:20	6:00	8.70	10.59	11.95	12.73	13.64
15	14:50	6:30	8.39	10.40	11.80	12.07	13.16†
Pasturage diet—unfistulated bull							
16	10:00		7.78	8.59	9.38	10.05	10.80
Pasturage diet—fistula steer							
17	14:45		10.01	10.92	11.69	12.35	13.01

* Initial pH, 6.2; final pH, 5.4.
† Initial pH, 6.4; final pH, 5.05.

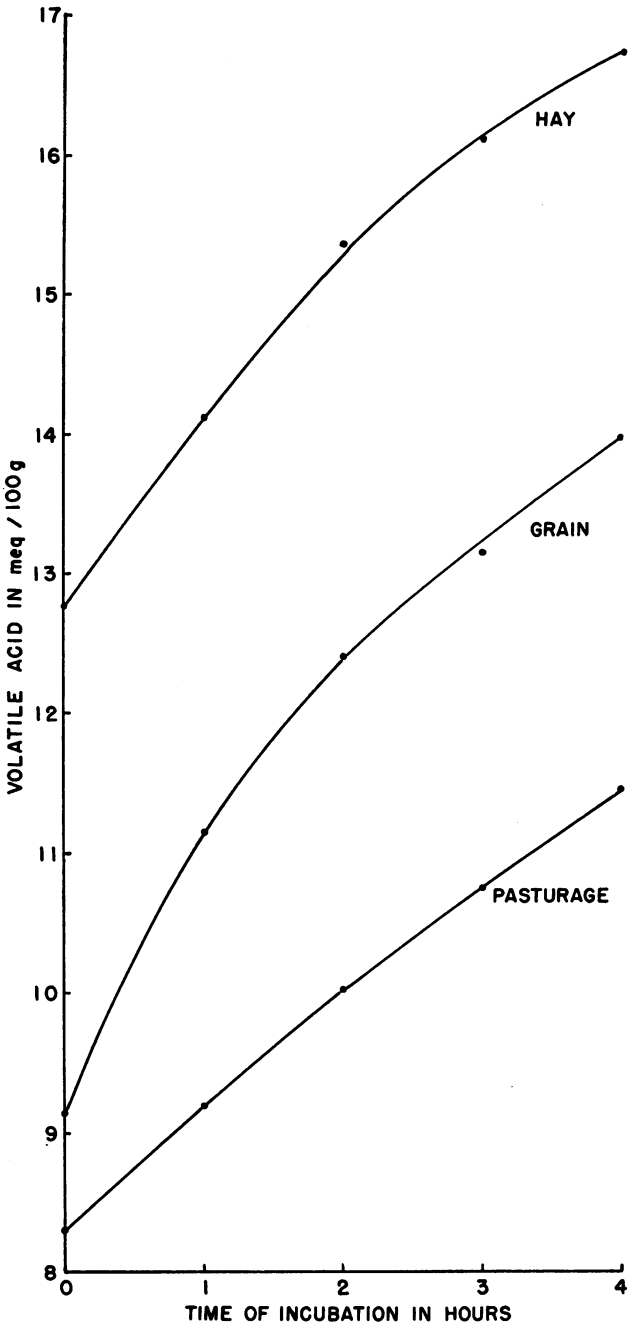

FIG. 3. Volatile acid in incubated hay-, grain- and pasturage-fed rumen contents.

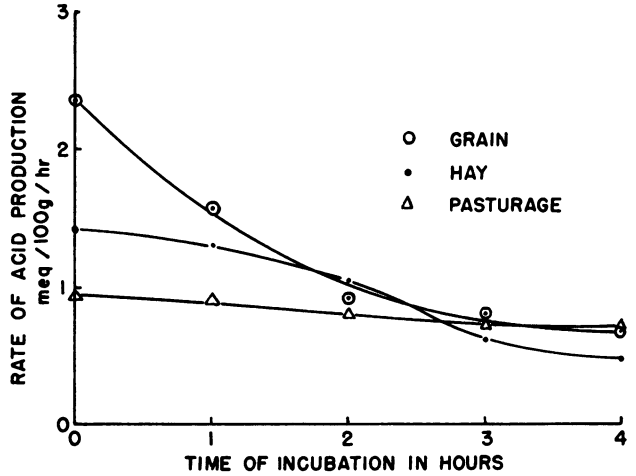

FIG. 4. Estimated rates of volatile acid production in incubated rumen contents.

of decrease in available substrate and inhibition by accumulation of fermentation products. The extent to which the rate in the rumen of hay-fed animals differs from that in the incubated cultures was estimated by determining the slope of the hay curve at various times, using the graphic method employed by Adams and Hungate (1950). The curves for the pasturage and grain experiments were similarly used to estimate rates. In figure 4 are plotted these estimated rates at zero, 1, 2, 3, and 4 hours, respectively, for each of the diets.

The points for the hay diet fit a fairly smooth curve in figure 4. The rate of volatile acid production is greatest initially and gradually decreases, in agreement with expectation. The estimated value of 1.42 mEq/100 g/hr at zero hour is only slightly greater than the average increase in volatile acids during the first hour as determined experimentally (1.37).

The relatively low increase in volatile acids in the pasturage runs may be explained by failure of the stomach tube procedure to obtain a representative sample insofar as solids content was concerned. Presumably, pasturage ingesta would resemble that from hay in having the major part of the microbial activity associated with the solids. The high increase during the first hour in the grain experiments is somewhat unexpected in view of the low solids content of these samples, but the greater value for volatile acid production as compared with hay is about that which would be expected from the usually greater gains of an animal on a grain diet. It is possible, due to the greater digestibility of starch, that the solids in the rumen samples of the grain experiments were sufficient to support microbial activity to the same extent as in the rumen.

B. Individual Volatile Acids

Since the various volatile acids differ in nutritional value, an attempt was made to determine the amount of each. In the first analyses, involving six of the runs on the hay-fed steer, the experimental error in separation and determination of the individual acids was deemed too great to permit evaluation of increases during the first hour and only the zero and 4-hour samples were analyzed. The results are shown in tables 9 and 10.

TABLE 9. *Amounts of individual volatile acids at zero and 4 hours in six experiments with hay-fed animal*

EXPERIMENT NO.	0 HR			PER CENT RECOVERY	4 HR			PER CENT RECOVERY
	ACETIC	PROPIONIC	BUTYRIC		ACETIC	PROPIONIC	BUTYRIC	
	mEq/100 g							
4	6.04	2.37	1.40	105	8.20	4.05	2.57	98
5	6.89	2.82	1.70	99	9.85	3.55	2.40	100
8	6.51	2.59	1.72	103	10.11	3.24	2.27	101
9	5.40	1.97	1.26	101	8.94	2.12	1.79	108
10	8.33	3.02	1.69	95	10.64	3.91	2.66	96
11	12.06	3.74	2.37	98	11.92	4.66	2.89	92
Mean...	7.54	2.75	1.69	100.2	9.94	3.59	2.43	99.2

TABLE 10. *Increase in individual volatile acids during 4 hours incubation of rumen contents from hay-fed animals*

EXPERIMENT NO.	ACETIC	PROPIONIC	BUTYRIC
	mEq/100 g		
4	2.16	1.68	1.17
5	2.96	0.73	0.70
8	3.60	0.65	0.55
9	3.54	0.15	0.53
10	2.31	0.89	0.97
11	−0.14	0.92	0.52
Mean increase.....	2.405	0.837	0.74
Standard error of mean increase.....	0.57	0.20	0.11
Percentage.....	60.3	21.1	18.6

The sum of the average increases in individual volatile acids (3.98/100 g) in these six experiments is about the same as the increase as determined by steam distillation (4.18 mEq). The slightly low value for the individual acids is probably due to an undetected error in the analysis for acetic acid at 4 hours in experiment 11.

If the percentage composition in which the volatile acids are produced at zero hour is the same as the proportion in which they are formed during the 4 hours of incubation, the analytical results for increased individual acids at 4 hours can be applied to the estimated zero rate. Although this identity cannot be tested experimentally, an indication of its validity can be obtained by comparing the composition of the acids produced during the first hour with that of the acids produced later.

A single run, in which individual volatile acids were determined at zero and 1 hour, was made with rumen contents from a fistula steer on a hay diet. The total increase in volatile acids was 1.12 mEq/100 g. The increase in individual acids was: acetic 0.73, propionic

0.25, and butyric 0.20, a total of 1.18 mEq/100 g/hr. These are in the proportions of 62 per cent acetic, 21 per cent propionic, and 17 per cent butyric acid, as compared with 60.3, 21.1 and 18.6 per cent, respectively, for the proportions of the volatile acids formed in the six hay experiments shown in table 10. Although, because of experimental error, these results from one run cannot be considered conclusive, they are at least in agreement with the assumption that during 4 hours of *in vitro* fermentation the ratios in which the individual volatile acids are produced do not change appreciably. Applying the above percentage composition to the estimated zero hour rate of 1.42 mEq/100 g/hr (figure 4) the average daily caloric values of the acids produced in 100 kg of rumen contents of the animal on the hay diet are: acetic = 4300, propionic = 2600, and butyric = 3200, a total of 10,100 calories.

In the experiments using rumen contents from the grain-fed animals, the individual volatile acids were determined at zero and 2 hours. In experiment 13 there was such a discrepancy between the recovery of volatile acid initially (108 per cent) and at 2 hours (93 per cent) that the results were not included in the calculations. The remaining two experiments gave results as shown in table 11.

The sum of the mean increases in individual volatile acids (2.87 mEq/100 g) in these two experiments is about the same as the increase found by steam distillation (3.16 mEq). The estimated rate of acid production for the grain experiments at zero time (figure 4) was 2.35 mEq/100 g/hour. At this rate, using the percentage composition shown in table 11, the energy in the acids available per day per 100 kg of rumen contents would be: acetic = 7450, propionic = 4760, and butyric = 4140, a total of 16,350 calories.

The curve for the grain experiments in figure 4 shows an initially high value with a more rapid decrease in rate than is evident in the curves for hay and pasturage. This may be due to an inhibition by the increased products from the more rapid fermentation (final pH was 5.1 to 5.4) or to a greater depletion of

TABLE 11. *Amounts of individual volatile acids at zero and 2 hours in two experiments with the grain-fed animal*

EXPERIMENT NO.	0 HR			PER CENT RECOVERY	2 HR			PER CENT RECOVERY
	ACETIC	PROPIONIC	BUTYRIC		ACETIC	PROPIONIC	BUTYRIC	
	mEq/100 g				*mEq/100 g*			
14	4.82	2.24	1.38	97	6.28	3.00	1.99	94
15	4.62	1.87	1.29	93	6.80	2.41	1.48	91
Mean	4.72	2.06	1.34		6.54	2.71	1.74	
Mean increase					1.82	0.65	0.40	
Standard error of mean increase					0.38	0.12	0.22	
Percentage composition of increased acid					63.4	22.6	14.0	

TABLE 12. *Amounts of individual volatile acids at zero and 1 hour in three experiments with animals on pasturage*

EXPERIMENT NO.	0 HR			PER CENT RECOVERY	1 HR			PER CENT RECOVERY
	ACETIC	PROPIONIC	BUTYRIC		ACETIC	PROPIONIC	BUTYRIC	
	mEq/100 g				*mEq/100 g*			
16	5.24	1.36	0.65	93	6.06	1.65	0.81	99
3	4.51	1.38	0.84	94	5.34	1.68	1.01	99
17	6.13	2.16	1.18	95	6.32	2.29	1.35	91
Mean	5.29	1.63	0.89		5.91	1.87	1.06	
Mean increase					0.62	0.24	0.17	
Standard error of the mean increase					0.21	0.05	0.01	
Percentage composition of increased acid					60.2	23.3	16.5	

substrate. Although the steam distillation values were more variable for the grain than for the hay and pasturage experiments, the relatively small standard error for the mean increase in steam distillation values between zero and 1 hour (2.01 $\pm$ 0.10) makes it improbable that the greater rate for the grain experiments is due to experimental error.

In the experiments with rumen contents from animals on pasturage the analyses for individual volatile acids were performed on the zero and 1-hour samples with results as shown in table 12.

The sum of the mean increases in individual volatile acids (1.03 mEq) is greater than the average increase in total volatile acids (0.91 mEq) but the difference is within the experimental error involved in the two analyses.

Using the zero hour rate of 0.95 mEq/100 g/hour (figure 4), the energy available upon oxidation of the acids produced each day per 100 kg of rumen contents in the animals on pasturage would be: acetic = 2870, propionic = 1950, and butyric = 2050 calories, a total of 6870 calories.

C. Relation of Volatile Acid to Substrate Fermented

In order to estimate the approximate amount of substrate required for the production of a given quantity of volatile acid, an experiment was run in which 430 mg of cellulose were added to 260 ml of sterile culture medium composed of 210 ml of inorganic salt solution and 50 ml of rumen fluid. Phosphate instead of bicarbonate buffer was used. A control contained no cellulose. The control and the cellulose culture were each inoculated with 10 ml of fresh rumen fluid, made anaerobic, and sealed in an all-glass culture flask. After 24 hours of incubation at 38 C all the cellulose had disappeared. After 48 hours both control and experimental flasks were sterilized in the autoclave and, after cooling, were opened and analyzed for volatile acid. The experimental flask contained 3.12 mEq more volatile acid than the control. Assuming that the ratio between substrate fermented and acids

produced was the same in this experiment as in the rumen, calculation shows that 4686 g or 10.35 pounds of feed per day would be fermented in producing 34 equivalents of volatile acid, the amount estimated to be formed in 100 kg of rumen contents of the hay-fed animal.

Discussion

The fact that in the hay and pasturage experiments, the increase in volatile acid during the second hour of incubation (tables 7 and 8) was almost as great as during the first hour, supports the assumption that the imposed culture conditions did not rapidly alter the rate of the rumen fermentation. This was further indicated by the negligible influence of dilution in experiment 3.

The error in estimating zero hour rate was greatest in the grain experiments. Steam distillation values at one-half-hour intervals would have been preferable in these runs and the relatively rapid increases in volatile acid would have made such determinations practicable.

The present studies have not disclosed any great differences in the intensity of fermentation in rumen contents removed at different times of day. This result does not necessarily contradict those (Phillipson, 1942; Hale *et al.*, 1947) in which a cycle of fermentative activity has been shown to follow ingestion of feed. Acid production per unit volume or weight of rumen contents can accurately reflect the total acid production only if the volume of rumen contents remains the same. Since the rumen volume is not constant the differences in total volatile acid production in a given animal during the day could be due to changes in the rumen volume rather than to different intensities of microbial activity per unit volume. Differences in the availability of feed at various times during the day also affect the uniformity of the fermentation.

To calculate the total production of volatile acids in the bovine animal it is necessary to make some estimate of the average volume of the rumen contents. A perusal of the literature shows widely varying reports of bovine rumen volume. Sisson (1914) lists values as high as 60 gallons (227 kg) for large animals down to 25 gallons for small animals. Elsden *et al.* (1946) found 54 kg of rumen and reticulum contents in a 364-kg steer and 36.8 kg in a 182-kg steer. Winogradowa-Fedorowa and Winogradow (1929) reported the volume of rumen and reticulum contents as ranging between 56 and 87 kg. Blamire (1952) obtained an average weight of 43.4 kg for the rumen plus reticulum contents of 11 mature cows. Eleven oxen averaged 36.5 kg, five heifers averaged 30 kg. and one bull contained 65 kg of rumen contents. The weight of the contents in all these animals averaged 68 per cent of the total weight of the rumen and reticulum. Hoflund (1940) found the weight of the combined rumen and reticulum of Swedish Red cattle to vary between 61 and 105 kg. If the contents averaged 68 per cent of the total weight, Hoflund's figures would indicate 41.4 to 71.4 kg as the weight of rumen contents, an average of 56.4 kg.

The values of both Blamire and Hoflund were obtained on abbatoir animals in which the rumen contents would be expected to be less in amount than in others more regularly fed. The breed and weights of the animals cited by Blamire were not reported and his values appear low if referred to a 500-kg animal. Mature animals of the breed studied by Hoflund weigh about 500 kg (Garm, 1949).

If the weight of rumen contents be assumed proportional to the three-fourths power of the weight of the animal, Elsden's figures give about 70 kg as the weight of rumen contents in a 500-kg animal. Assuming that Winogradow's average value of 71.5 kg referred to an average weight of 500 kg for the whole animal, these two estimates agree fairly well. They are somewhat higher than the values of Hoflund for abbatoir animals, but lower than the volumes reported by Sisson.

If 70 kg be taken as the average weight of rumen contents in a 500-kg cow, the total calories available to the animal by oxidation of the volatile acids formed in the rumen can be calculated from the results of the present investigation. A hay-fed animal would obtain 7,070 calories, a grain-fed animal 11,450 calories, and a grass-fed animal 4,810 calories, through oxidation of the volatile acids. Armsby and Moulton (1925) estimated the maintenance requirement of a 1,000-lb bovine as 7,300 calories. Assuming that the energy requirements are proportional to the three-fourths power of the weight (Kleiber, 1947), a 500-kg bovine would require 7,850 calories per day for maintenance.

Exact nutritional data were not available for all the animals used in the present experiments. The fistulated animal used in the hay runs showed an average daily gain in weight of 0.75 lb. Its weight at the time of the experiments was 500 kg. If the available energy in its food be estimated at 10,800 calories, the calculated caloric value of the volatile acids produced in this animal is 66 per cent of the total.

Using the results of the *in vitro* experiment in section C, showing that 10.35 lb of feed would be fermented in the production of 34 equivalents of volatile acid, the quantity of substrate fermented in a 500-kg animal would be expected to be about 7.25 pounds. The usual total digestable nutrients (TDN) requirement for maintenance of a 500-kg animal is about 8.5 pounds (Bull and Carroll, 1949). Since the animal gained some weight during the period of experimentation its nutrient supply was greater than 8.5 pounds. Assuming that 2.5 lb of digestible nutrients above maintenance level were needed for the weight increase of 0.75 lb per day, the TDN ingested by the hay fed animal would be

11.0 lb. The volatile acids from the rumen fermentation would require for their production about 66 per cent of the TDN.

The grain ration of the unfistulated bull was designed for fattening. If the caloric value of its feed be estimated at 16,000 calories, the volatile acids from the rumen fermentation would meet 71 per cent of the total. Although in the pasturage experiments the inadequacy of the sampling technique gave low values, the calculated rate of the volatile acid production would meet 61 per cent of the maintenance requirement.

The agreement between the estimates of the relative importance of the rumen fermentation for the hay and grain experiments is fairly good and it may be tentatively concluded that the volatile acids produced in the rumen account for about 70 per cent of the total energy requirement. The mechanisms by which the remaining requirement is met presumably include 1) digestion of protein and other materials in the abomasum, 2) fermentation in the large intestine, and 3) formation in the rumen of additional products such as lactic and succinic acids. The chief errors in the calculated values arise: 1) in obtaining representative samples of rumen contents, a source of error emphasized by Pearson and Smith (1943); 2) in the estimation of average rumen volume; and 3) in the assumption of an average fermentation rate, an error partially met by making repeated runs and by determining rates at various times of day.

The average proportions in which the individual acids were produced from the various feeds were quite similar. This may be a coincidence since the standard error in determination of the separate acids is relatively large. Card and Schultz (1953) reported that the amounts of the individual volatile acids in rumen contents of animals on various diets disclosed small but significant differences.

In the six hay experiments the percentages in which the various volatile acids occurred in the zero hour sample were: acetic 62.9, propionic 23.0, and butyric 14.1. The acetic acid formed in 4 hours constituted 32 per cent of that initially present, the propionic acid 30.4 per cent, and the butyric acid 43.7 per cent.

In the two grain experiments the initial percentages were: acetic 58.1, propionic 25.4, and butyric 16.5. The acid formed in 2 hours constituted the following percentages of the amount of each initially present: acetic acid 38.5, propionic acid 31.5, and butyric acid 30.0.

In the three pasturage experiments the initial percentages were: acetic 67.8, propionic 20.8, and butyric 11.4. The acetic acid formed in 1 hour was 11.7 per cent of that initially present, propionic acid 14.7 per cent, and butyric acid 19.1 per cent.

The results suggest that more extensive investigations using this method might disclose significant differences in the proportions of the volatile acids produced on the different diets, but the error in analysis is so great that the results of the present experiments cannot be regarded as conclusive.

In the above data there is a negative correlation between the relative rate of production of a particular acid and the initial proportion in which that acid occurs. In the pasturage experiments in which the acetic acid was initially 67.3 per cent of the total volatile acid, its increase was relatively less than that of propionic or butyric acid. In the grain experiments in which acetic acid constituted only 58.1 per cent of the initial acid, its relative increase was greater than for either propionic or butyric acids. The data for the hay experiments are consistent with these trends. The results suggest that the greater the proportion in which a particular acid is present, the less the proportion in which it is produced.

It is highly probable that the accumulation of undissociated volatile acids is an important factor causing a diminution of volatile acid production with time. In experiment 13 with the grain-fed animal, the initial pH of rumen contents was 6.2 and the pH after 4 hours of incubation was 5.4. In experiment 15 the initial pH of 6.4 fell to 5.05 after 4 hours. During this change in pH the rates of volatile acid production fell from 2.35 mEq at zero hour to 0.70 at 4 hours (figure 4). If accumulation of undissociated volatile acids is effective in inhibiting the total fermentation it seems possible that changes in the proportions of individual acids may influence their relative rates of production.

It should be mentioned that the theoretically most accurate method for ascertaining the importance of the individual volatile acids would be to construct for each a curve such as those shown in figure 3 and to determine graphically the zero hour rate. The sum of the zero hour rates for the individual acids should equal the zero hour rate for total volatile acid. The magnitude of the analytical work needed to provide curves with sufficient accuracy to permit reliable estimates of zero hour rates placed this approach outside the scope of the present investigation.

The purpose of the present studies was more to explore the method than to provide a high degree of statistical accuracy. Additional experiments on numerous animals, preferably in different geographic locations, together with information on rumen volumes, will be needed to establish more accurate norms for the rumen fermentation in animals on various types of feed. The results obtained thus far do support the hypothesis that the major part of the bovine energy requirement is met by the volatile acids produced in the rumen and encourage the belief that the method has potential value in analyzing the physiology of food utilization in the ruminant.

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Summary

The rate of volatile acid production in the bovine rumen was estimated by constructing a curve showing total volatile acid at various times in rumen contents incubated under conditions simulating those of the rumen. The slope of the curve at zero hour, that is, the rate of production of volatile acid in the rumen, was calculated. The volatile acids were separated into acetic, propionic, and butyric fractions and the rate of production of each of these was estimated.

The most information was obtained for animals on a hay diet but three runs were made on grain-fed and grass-fed animals, respectively. The grain-fed animals showed the highest rate of production of volatile acid, the hay-fed were intermediate, and the pasturage animals showed the lowest rate. The low values for pasturage can be accounted for by failure to obtain representative rumen samples. By estimating the average rumen volume a rate of production for the animal was calculated. The energy available from the fermentation acids was found to be about 70 per cent of the estimated total energy requirement.

The largest sources of error in the estimations were the sampling of rumen contents, the estimation of average rumen volume, and the variation in fermentation rate with the time of day.

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