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STUDIES OF VARIATION IN pH AND VOLATILE
FATTY ACID CONCENTRATION WITHIN THE
RETICULUM AND RUMEN OF A GRAZING COW

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INTRODUCTION

The ruminant is but one component of the great cycle of energy utilization, a cycle which derives its energy from that of the sun and enables man not only to exist, but also to flourish.

The complex and comprehensive nature of the cycle does not belittle the role played by ruminants - the conversion of high fibre feedstuff of no direct value to man into a whole array of products, many of which are now considered essential to human nutrition and welfare.

Their ability to perform this feat is attributable to the symbiotic relationship which exists between the microorganisms of the rumen and the host animal. This remarkable example of symbiosis has always elicited man's interest even though the interest initially arose from an inherent curiosity rather than a specific need.

However, over the last few decades the resources of workers from diverse fields of science have been directed towards an understanding of the activities within the rumen and their importance to the nutrition of the host animal. This upsurge of interest has arisen from the demand for more efficient agricultural production which, from the animal side, can be achieved by maximizing the efficiency with which the ingested food material is converted to the animal products. Inherent in this concept is the prevention and correction of the metabolic disorders which tend to accompany the intense and unnatural exploitation of the ruminant.

The outcome is that many of the activities within the rumen are no longer a mystery to the biologist and chemist.

Thus it has been shown that the amounts and proportions of the individual volatile fatty acids produced within the rumen vary according to the diet. The

amount and proportions of these acids have also been shown to influence the value of the food in meeting the requirements of maintenance, growth and fattening. Similarly, in the lactating cow, they influence the yield and composition of the milk.

Most of this work has been done in countries where the reliance on direct consumption of pasture by grazing animals is less than in this country. The result is that New Zealand workers have been faced with the study of problems that are not so important elsewhere. Although a considerable amount of local work has been done, particularly on some metabolic disorders and deficiencies, much still remains. Very little information is available concerning the effect on the fermentation processes of feeding pastures of varying botanical and chemical composition and the different grazing practices used in their utilisation. The normal variability in the concentration of fermentation end products that can be expected with grazing animals is particularly lacking in definition.

In the programme of work originally planned, it was hoped that some information on the normal state of microbial activity in the rumen of the grazing animal would be obtained. However, the literature revealed that one of the features of the rumen contents is a marked heterogeneity in the distribution of ingested food materials. Furthermore, a few reports indicated that in the bovine rumen this heterogeneity was reflected in the fermentation end products in the form of concentration gradients or stratification. The impression was gained that this effect may be particularly marked in grazing animals.

Thus, although the analytical work involved limited the scope of the study, an attempt was made to obtain a clearer understanding of the nature and magnitude of this stratification. Such a study was believed necessary for the intelligent selection of a sampling site in any studies on the microbial activity within the rumen of the grazing bovine.

Two feeding systems were chosen. The first involved a limited period of grazing after a period of fasting, a system often used in comparing the effect

of different diets on the fermentation processes. The second system consisted of free grazing similar to that which would prevail in any survey work.

In preliminary trials it was found that in some instances an analytical procedure commonly used for the determination of volatile fatty acid (VFA) concentration in rumen liquor failed to give reproducible results. Differences in VFA concentration of up to 30% were found between determinations on the same sample of rumen liquor. Further, the number of rumen samples in the experiments as planned necessitated their storage for a period of several days. It was therefore deemed advisable not only to compare some of the commonly used procedures for determining the VFA concentration in rumen samples but also to examine the stability of the VFA's during storage.

Part I is a report of this study and although it is complete in itself it is considered an integral part of the whole thesis as entitled. Each feeding system is dealt with separately in Chapters II and III of Part II. In Chapter IV the pertinent findings are discussed in relation to possible causes of stratification and the effect it has on sampling the rumen contents. A general summary of the results and conclusions for both experiments is presented in the final chapter.

PART I

ESTIMATION OF THE VOLATILE FATTY

ACID CONCENTRATION BY STEAM

DISTILLATION

1 REVIEW OF LITERATURE

Recognition of the full importance of volatile fatty acids (VFA) in ruminant nutrition was not made possible until the development of reliable methods for the analysis of VFA mixtures. Following the introduction of partition chromatography by Martin and Synge (1941), several reliable column partition chromatographic techniques have been reported (Elsden, 1946; James and Martin, 1952; Wiseman and Irvin, 1957).

Before the chromatographic procedure can be applied to the separation and identification of VFA in biological fluids, their isolation from other acidic substances is generally required. This isolation is most commonly achieved by steam distillation; titration of the distillate giving a measure of the total volatile acids. Methods that eliminate the need for steam distillation have been developed (Neish, 1949; Wiseman and Irvin, 1957).

As many organic acids other than fatty acids are volatile in steam but to a lesser extent than the lower fatty acids, the conditions of distillation are adjusted in an endeavour to eliminate from the distillate, all but the fatty acids.

Of the numerous procedures available, the double distillation method of Friedemann (1938) has proved the most reliable. In the second distillation, use of mercuric salts and magnesium sulphate quantitatively removes pyruvic acid, reduces lactic acid to less than 0.2% of the quantity in the original sample and oxidises formic acid (Baker, 1957).

McAnally (1944) found that with blood, the method of Friedemann gave results that were dependent on the time taken for distillation indicating that decomposition of labile substances had occurred. Precipitation and subsequent removal of protein from a diluted sample of blood, besides giving a good

recovery of added acids, gave a result unaffected by time taken for distillation. This method, like that of Friedemann's, is based on the observations of Olsted, Whitaker and Duden (1929) that high concentrations of $\text{Mg}\cdot\text{SO}_4\cdot 7\text{H}_2\text{O}$ greatly increases the rate of distillation of the VFA. This method has been applied to the analysis of rumen fluid by many subsequent workers, the essential steps being the precipitation and removal of protein, acidification and steam distillation in the presence of $\text{Mg}\cdot\text{SO}_4\cdot 7\text{H}_2\text{O}$.

In rumen fluid where the levels of lactic and pyruvic acids are considered negligible under normal conditions, the simpler and less tedious method of McInally is usually preferred to the accurate, but time consuming method of Friedemann. Many modifications to these methods have been reported (Opperman et al, 1957; Johns, 1955; Barnett and Reid, 1957). The most widely used steam distillation apparatus is that of Marthen (1942).

II MATERIALS AND METHODS

A large volume (2 l.) of rumen liquor was collected from a fistulated cow by expressing a portion of the rumen ingesta through a single layer of cheese cloth. Representative samples of the liquor were treated as follows.

Procedure A (Johns, 1955). Preserved by addition of 2% (V/V) HgCl_2 as a saturated solution. Aliquots were distilled in the presence of 1ml. 10N H_2SO_4 saturated with $\text{Mg}\cdot\text{SO}_4\cdot 7\text{H}_2\text{O}$.

Procedure B. As for Procedure A but the rumen liquor was divided into eight portions (one portion analysed each day) and stored at -10°C .

Procedure C. 20 ml. 10N H_2SO_4 were added in the proportion of 20 ml. to each 100 ml. of rumen liquor. The resulting suspension was stored and used for subsequent analysis.

Procedure D. As for Procedure C except that immediately prior to distillation, an aliquot was centrifuged at 500 g. to remove most of the particulate material. The resulting supernatant was used for analysis.

Procedure E (Annison, 1954). The sample was mixed with an equal volume of N H_2SO_4 saturated with $\text{Mg}\cdot\text{SO}_4\cdot 7\text{H}_2\text{O}$, allowed to stand for 30 minutes at room temperature to denature protein which was then removed by centrifuging at 500 g. for 5 minutes. The supernatant was stored for subsequent analysis.

Procedure F (Balch and Rowland, 1957). The sample was centrifuged (500 g. for 5 minutes) to remove suspended solids and the supernatant diluted with four volumes of water. Equal volumes of diluted fluid and N H_2SO_4 saturated with $\text{Mg}\cdot\text{SO}_4\cdot 7\text{H}_2\text{O}$ were mixed and left overnight to denature the protein. The precipitate was centrifuged off and the supernatant stored for analysis.

To determine the recovery of the VFA's, the same procedures were applied to further samples of rumen liquor to which was added a known amount of a standard solution containing 10.22 mM/100 ml. of acetic, propionic and butyric acids in the proportions 6 : 3 : 1 :

With the exception of Procedure B, storage was at 5°C. On each of eight successive days, the VFA concentration in samples representing all procedures was determined in duplicate. The order in which the ten samples were analysed was randomised by drawing ten numbered marbles from a bag.

5 ml. aliquots were distilled in a Markham still. Two successive 50 ml. fractions of distillate were collected and titrated with 0.06N NaOH using phenolphthalein as indicator. During titration of the 50 ml. fractions, interference by CO₂ was reduced by using a sintered glass filter stick to bubble CO₂ free air through each fraction. Use of 0.06N NaOH obviated the extreme care required when dealing with more dilute (< 0.02N) alkali.

TABLE II

THE CONCENTRATION OF VFA IN THE RUMEN LIQUOR AFTER
THE ADDITION OF 25 ml OF A STANDARD SOLUTION OF VFA
(10.22 mM/100 ml) TO EACH 100 ml OF RUMEN LIQUOR.

METHODS AS FOR TABLE I (mM/125 ml)

		PROCEDURE					
		A	B	C	D	E	F
Day 1		15.8	15.7	16.2	16.0	15.9	12.9
		15.8	15.7	16.1	15.9	15.9.	12.8
2		15.7	15.7	16.2	15.9	15.9	14.7
		15.7	15.6	16.0	15.9	15.9	14.8
3		15.7	15.8	16.0	16.0	15.7	13.2
		15.7	15.8	16.1	16.0	15.7	13.2
4		15.7	15.7	16.0	16.0	15.8	13.6
		15.7	15.7	16.0	16.0	15.8	13.6
5		15.7	15.7	16.0	16.0	15.7	13.4
		15.7	15.8	15.9	16.0	15.8	13.3
6		15.7	15.8	16.0	16.0	15.9	13.3
		15.7	15.8	16.0	16.0	15.9	13.3
7		15.7	15.8	16.0	16.0	15.8	13.3
		15.7	15.8	15.9	16.0	15.9	13.3
8		15.7	15.7	16.0	16.0	15.8	13.4
		15.7	15.7	16.0	16.0	15.8	13.6

ANALYSIS OF VARIANCE

SOURCE	d.f.	M.S.	F.	
Procedures	4	0.3180	42.4	**
Days	7	0.0043	< 1	
Interaction	28	0.0075	5.00	**
Determinations	40	0.0015		

** (P < 0.01) Highly significant

Data for Procedure F excluded from statistical analysis

TABLE 1

THE VFA CONCENTRATION IN RUMEN LIQUOR ON EACH OF EIGHT
SUCCESSIVE DAYS AS DETERMINED IN DUPLICATE BY FIVE PROCEDURES
(mM/100 ml)

	PROCEDURE					
	A	B	C	D	E	F
Day 1	13.2	13.3	13.6	13.5	13.3	11.6
	13.2	13.3	13.6	13.5	13.3	11.7
2	13.1	13.3	13.6	13.4	13.3	11.0
	13.2	13.3	13.6	13.6	13.3	11.0
3	13.2	13.3	13.6	13.5	13.2	11.0
	13.3	13.3	13.6	13.6	13.2	11.0
4	13.2	13.0	13.5	13.5	13.2	11.1
	13.2	13.0	13.5	13.5	13.3	11.2
5	13.2	13.2	13.6	13.6	13.2	11.1
	13.2	13.2	13.6	13.5	13.3	11.4
6	13.2	13.3	13.5	13.6	13.3	11.7
	13.2	13.3	13.5	13.6	13.3	11.7
7	13.2	13.3	13.5	13.6	13.3	10.8
	13.2	13.3	13.5	13.6	13.3	10.8
8	13.1	13.3	13.6	13.6	13.2	11.1
	13.1	13.3	13.6	13.6	13.2	11.0

ANALYSIS OF VARIANCE

SOURCE	d.f.	M.S.	F.	
Procedures	4	0.515	68	**
Days	7	0.010	1.33	NS
Interaction	28	0.0075	6.00	**
Determinations	40	0.00125		

NS Not significant (P > 0.05)
** (P < 0.01) Highly significant

Data for Procedure F excluded from statistical analysis

III RESULTS

The VFA concentration in the rumen liquor, and rumen liquor plus added VFA, for the eight days are presented together with the analyses of variance in Tables I and II respectively. Because of their obvious inconsistency, the results obtained by using Procedure F were excluded from the statistical analyses.

Similar conclusions were drawn from the results of both analyses of variance. The significant interactions indicate that with some Procedures, there was a significant difference in VFA concentration between days. The difference required to reach significance at the 5% level was 0.17 mm M/100 ml. for both tables. The significant interaction was apparently caused largely by the VFA concentration as determined by Procedure B on Day 4 (Table I) and the concentration as determined by Procedure C on Day 1 (Table II). For any one procedure there was no trend in VFA concentration with days. On the basis of these observations it was concluded that for Procedures A, B, C, D and E, no change of practical importance occurred during the eight days of storage.

The Procedure means for both sets of VFA concentration determinations are presented in Table III together with standard errors and the detectable differences required for significance at two levels.

TABLE III

The means of the VFA concentration of rumen liquor with and without added VFA over a storage period of eight days as determined by five procedures.

		Procedure	Mean	General Mean	S.E.	Difference required	
						d0.05	d0.01
Without added VFA	A		13.19	13.36	± 0.02	0.06	0.08
	B		13.25				
	C		13.56				
	D		13.55				
	E		13.26				
With added VFA	A		15.71	15.86	± 0.02	0.06	0.08
	B		15.74				
	C		16.03				
	D		15.98				
	E		15.83				

This table illustrates that Procedures C and D gave higher estimates of VFA concentration than any of the other procedures.

The analysis of variance of the recovery data (Table IV) showed that the procedures did not differ in their ability to recover added VFA's. The average percentage recovery of 96% was considered satisfactory.

TABLE IV

THE CONCENTRATION OF THE STANDARD SOLUTION OF VFA ADDED TO THE RUMEN LIQUOR AS CALCULATED FROM THE AVERAGES OF DUPLICATE DETERMINATIONS IN TABLES I AND II BEFORE THEIR CORRECTION TO ONE DECIMAL PLACE. (TRUE CONCENTRATION-10.22 mM/100 mL)

(mM/100 mL)

PROCEDURE

	A	B	C	D	E	F
Day 1	10.3	10.0	10.2	9.9	10.7	4.8
2	10.4	9.5	10.0	9.6	10.4	15.0
3	10.0	10.2	10.0	9.8	10.0	8.4
4	10.0	11.1	9.8	10.0	10.0	9.8
5	10.0	10.4	9.4	10.0	10.0	8.4
6	10.0	10.0	10.2	9.8	10.4	6.4
7	9.8	10.0	9.8	9.8	10.3	10.0
8	10.2	9.4	10.0	9.8	10.4	9.8

ANALYSIS OF VARIANCE

SOURCE	d.f.	M.S.	F.	
Procedures	4	0.2275	2.21	NS
Days	7	0.0586	0.57	NS
Error	28	0.1029		

NS Not significant ($P > 0.05$)

Data for Procedure F excluded from statistical analysis

IV DISCUSSION

The procedure of Balch and Rowland (1957) proved unsatisfactory when used under the existing experimental conditions. As this procedure involved dilution of the rumen liquor, the titration of the steam distillates required only small volumes of 0.06N alkali (approx. 1 ml.). For this reason it was considered that the unsatisfactory results obtained were a reflection of the relative magnitude of the titration errors rather than an indication of an inherent fault in the procedure.

The procedures did not differ in their ability to recover added VFA's but there was a significant difference in their estimation of the initial VFA concentration in the rumen liquor. However, the percentage difference between the mean estimates (Table III), suggests that although the difference was statistically significant, it may not be of practical importance, for in rumen studies, sampling errors are usually large (Williams and Christian, 1956).

It was concluded that the VFA concentration of the rumen liquor, as estimated by five procedures did not show an alteration of practical importance over the storage period of eight days. This finding appears incompatible with the inconsistent results that were obtained using Procedure A in preliminary trials. However, the results reported here provide no grounds for assuming that any of the five procedures would give similar results on other rumen samples. The observation was made during the preliminary trials that samples which failed to give consistent results with Procedure A, gave consistent results with Procedure B. Besides indicating that it was unlikely that it was the experimenter at fault, this also suggests that a Procedure X Sample interaction may occur.

In view of this, it was concluded that for subsequent work, procedures involving denaturation and removal of protein would be the least likely to cause

trouble. Of the two appropriate procedures, the higher titre required in titration of the Procedure D distillates, makes this method preferable to Procedure E.

In ascertaining the validity of methods for determining VFA concentration in biological fluids, complete recovery of added VFA serves as a valuable check on possible losses associated with deproteinization and distillation procedures provided the assumption is accepted that the recovery of VFA originally present is as good as that of the added VFA. However, doubt still remains as to the nature of the acidic substances estimated.

The lower monohydroxy and keto acids such as lactic and pyruvic acids are slightly volatile with steam (Baker, 1957). Decomposition to labile acidic substances may result in other acidic substances occurring in the distillate.

Lactic acid is the only non-fatty acid likely to be present in appreciable amounts in rumen liquor. Although usually present in trace amounts only, enhanced levels are encountered in the rumen when excessive amounts of readily fermented carbohydrates are consumed by the animal (Balch and Howland, 1957; Briggs et al, 1957). Even in such cases, it was accepted by these authors that a sufficiently accurate estimate of the VFA concentration was obtained by use of the simple steam distillation procedures similar to those outlined above.

That some error will be present is shown by the work of Elsdon (1946) and Reid and Lederer (1951).

In order to determine what portion of the titratable acidity was due to fatty acids with the exception of formic, Elsdon performed a series of analyses on sheep rumen fluid in which the steam distillates obtained using the procedure of McNally were collected after titration and re-distilled according to the method of Friedemann. On the assumption that the acids collected by the latter procedure were fatty acids only, the method of McNally over-estimated the VFA

concentration by 10-15%, a figure that has possibly been reduced since the introduction of the Markham still and a blank titration.

Reid and Lederer devised a paper partition chromatographic procedure for the separation, identification and estimation of individual VFA from C₂ - C₇. As formic and lactic acids could not be separated from acetic acid by this method, their removal by preliminary steam distillation was necessary. They found however, that with rumen liquor containing up to 5 m.mole/100 ml. of lactic acid, the amount of lactic acid which distilled over when using McNally's method caused an error of only 3 - 5% in the determination of acetic acid. The error caused by a single distillation can therefore be neglected in all instances except those where unusually high levels of lactic acid (e.g. Briggs et al, 1957) are encountered.

Thus it is concluded that the high levels of VFA present in the ruminal contents can, for routine analysis, be estimated sufficiently accurately by the steam distillation procedures outlined. However, in view of comments made above, use of procedures that involve the removal of protein may be advisable. More accurate but time consuming procedures similar to those used for blood are obviated by the high levels of VFA and low levels of interfering substances found in the rumen.

V SUMMARY AND CONCLUSIONS

1. A preliminary investigation of the stability of the VFA concentration as determined by steam distillation was carried out on stored rumen liquor samples. Six procedures of preparation were used and their abilities to recover added VFAs were compared. Duplicate determinations for each sample were performed on each of eight days.
2. One procedure gave variable estimates of the VFA concentration of the rumen liquor.
3. The remaining five procedures differed significantly in their abilities to estimate the initial VFA concentration of the rumen liquor. It was suggested that this difference may not be of practical importance.
4. All procedures except one did not differ in their abilities to recover added VFAs. The recoveries achieved were considered satisfactory.
5. No change of practical importance was found in the VFA concentration during the eight day storage period.
6. It was concluded that the VFA concentration of rumen liquor could be determined with an accuracy suitable for routine analysis by the procedures outlined. However, certain reservations were made.

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