Degradation of Protein in the Rumen of the Sheep

1. SOME VOLATILE FATTY ACIDS, INCLUDING BRANCHED-CHAIN ISOMERS, FOUND *IN VIVO*

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Work has been in progress here for some years on the use made by the sheep of different kinds of protein under different conditions (to be published; for preliminary communications on certain aspects see Cuthbertson & Chalmers, 1950; Chalmers & Synge, 1950). The results obtained have laid stress on the degradation of protein nitrogen to ammonia in the rumen. The possible importance of this was minimized in a review article by Schieblch (1929) and seems to have been first explicitly recognized by Pearson & Smith (1943) on the basis of *in vitro* incubation of protein with rumen contents. Detailed physiological data, however, were first obtained by McDonald (1948*a, b*) who measured the concentration of ammonia in the sheep's rumen at different times after feeding various proteins, and also showed that appreciable quantities of this ammonia may be absorbed into the blood draining the rumen; the ammonia would then presumably to a large extent be converted to urea in the liver and so be excreted. McNaught & Smith (1947) and Elsdén & Phillipson (1948) have reviewed nitrogen metabolism in the rumen.

The studies made at this Institute have in fact shown that nitrogen retention and growth in sheep on various diets can be correlated inversely with the extent of ammonia production in the rumen. In parallel with this work it seemed desirable to try to find out what other products of protein breakdown are formed besides ammonia. The present paper describes some relevant *in vivo* phenomena observed by chemical analysis of samples of rumen liquor obtained from sheep in the course of experiments on protein utilization by Dr Margaret Chalmers and Dr R. L. M. Synge. The subsequent paper describes *in vitro* experiments with the mixed micro-organisms of such rumen liquors, which throw light on some of the chemical reactions undergone by protein during its degradation in the rumen. This work formed the subject of a Thesis submitted to Aberdeen University for the Ph.D. degree in 1951 and some of it was included in a preliminary communication (el-Shazly & Synge, 1950).

It seemed probable, from information available

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on bacterial deamination of amino-acids (cf. el-Shazly, 1952), that volatile fatty acids (VFA's) might be formed at the same time as ammonia. The higher members of the normal series present in the rumen (C_4 and higher) might be expected to arise to a significant extent from amino-acids as well as from carbohydrates; members of the branched-chain series, arising from the amino-acids valine, leucine and isoleucine, which usually contribute about 20% of the nitrogen of proteins, would not be expected to arise at all from carbohydrates. When this work was begun in 1948, none of the micro-methods available for analysing VFA's gave a good separation of branched- and straight-chain isomers. Some of the C_4 and (C_4 and higher) fractions isolated by the procedure of Elsdén (1946) were therefore subsequently examined by the oxidation method of Kline (1934, 1938). The results so obtained, though not inconsistent with the more recent findings, are not given here since the gas-liquid partition chromatogram of James & Martin (1951, 1952) became available later; this permitted unequivocal resolution of branched- and straight-chain isomers and of C_6 from C_4 and higher homologues, as well as being both rapid and convenient in use.

METHODS

VFA's used for control purposes were A.R. or 'laboratory reagent' quality (British Drug Houses Ltd.). They had the calculated equiv. wt. on titration with alkali and were homogeneous by the chromatographic methods mentioned below.

Rumen liquor. Samples were taken by Dr Margaret I. Chalmers, Dr A. T. Phillipson or Dr R. L. M. Synge from Cheviot ewes having permanent rumen fistulae, by aspiration through a glass or Polythene tube. The samples were strained through muslin and cooled quickly to 0°. The fistula operations had been done by Dr A. T. Phillipson.

Determination of ammonia in rumen liquor. 5 ml. of strained liquor and 5 ml. 0.2N-HCl were mixed, diluted to 50 ml. with water and filtered (cf. McDonald, 1948*a*). NH_3 was determined in the filtrate according to Conway & O'Malley (1942), adding 0.5-1.0 ml. of filtrate to 1 ml. saturated aqueous K_2CO_3 in the outer chamber of the diffusion cell.

Preparation of rumen liquor for analysis of carboxylic acids. In view of the special interest in the C_4 and higher VFA's, and other acids which might be retained by particles of the rumen liquor on their removal, tests were made comparing

the recovery of hexanoic acid added to rumen liquor: (a) after dialysis at rumen pH where diffusion would be as the anion; (b) on precipitation of particles, protein etc. by $\text{MgSO}_4\text{-H}_2\text{SO}_4$ according to the general procedure of McAnally (1944).

Portions (28–30 mg.) of hexanoic acid (as Na salt) were added to 19–25 ml. portions of rumen liquor. Control portions of rumen liquor without added hexanoate were treated in what follows in the same way. (a) The samples were dialysed in cellophan sacs at 0° for 36 hr. with intermittent stirring against sufficient distilled water to give a total vol. of 100 ml. (b) Water (25 ml.) was added to the samples and then 50 ml. of 2.5% (v/v) H_2SO_4 in sat. aqueous MgSO_4 . After keeping overnight at 0° the mixture was filtered. Known volumes of (a) diffusate or (b) filtrate were analysed for (C_6 and higher) VFA's by Elsdén's method after distillation or extraction (see below). The differences found, recalculated to the original samples, were taken to represent the recovery of added hexanoic acid, which was (a) 83–88%; (b) 83%. It might be expected that VFA's lower than C_6 would show higher recoveries. Recovery experiments with added *n*-butyric acid by method (b) gave, in fact, values in the range 82–102%. Accordingly rumen-liquor samples were prepared for analysis throughout the work according to method (b). The recoveries of the acids originally present may not have been as good as those of the added acids, but the results should at least have significance for comparative purposes. For obtaining VFA's the filtrates were distilled with steam in all-glass apparatus and successive portions of distillate were titrated with NaOH until no more acid distilled. Some determinations of total acids extractable by ether from the filtrate were made using a continuous liquid-liquid extractor. As well as giving good recoveries of VFA's, control experiments showed good recoveries of added succinic acid; it is apparent from inspection of the data collected by Washburn (1928) that several other well known physiologically important polycarboxylic acids, hydroxy acids, keto acids etc. would, if present, have been recovered in fair yield. However, on removal of VFA's by steam distillation from these ether extracts of rumen-liquor filtrates it was found that the non-volatile ether-extractable acids never exceeded 2.5% (equiv. basis) of the total VFA present.

In view of their favourable partition coefficients extraction by ether was sometimes used instead of steam distillation for convenience in isolating the (C_4 and higher) VFA's when it was wished to determine only these components. The ether extracts were titrated with aqueous NaOH, the ether was removed by evaporation and the residues were analysed in the same way as the steam distillates. McClymont (1951) has used a similar procedure.

Determination of VFA's by liquid-gel partition chromatography. The Na salts were prepared for chromatography and analyses were carried out as described by Elsdén (1946). VFA's of group (C_6 and higher) were sometimes not easily separated from the immediately following C_4 band, but no uncertainty attaches to the totals for (C_4 and higher) VFA's.

Determination of VFA's by gas-liquid partition chromatography. This was done as described by James & Martin (1952) using a 4 ft. column of internal diameter 4 mm.; 0.1 parts by weight of stearic acid were added to the silicone and the column was operated at 137° with manual control of the recording microburette. The pressure of N_2 was adjusted

to give 15 ml./min. of effluent gas. Solutions of mixed Na salts obtained as described above were concentrated to approx. 2N. Portions of this solution (30 μl .) were then pipetted on to a plug of pyrophosphoric acid-kieselguhr mixture in a short tube of the same bore as the gas-liquid chromatogram. The proportion of kieselguhr was adjusted so that the mixture stayed powdery on adding the liquid. More of the same mixture was then packed behind the sample, and the short tube was connected (rubber sleeve over close-fitting glass ends) to the inlet end of the gas-liquid chromatogram. By heating the plug tube and joint to 100° and applying vacuum to the outlet end of the gas-liquid chromatogram (which was maintained at room temperature) the free VFA's were distilled into the chromatogram tube as an anhydrous mixture. The chromatogram tube was then placed in its vapour jacket and the analysis was conducted without delay. This procedure was used by James & Martin (private communication) at one stage in their development of the method, but is now superseded (James & Martin, 1952). Aggregate recoveries of control mixtures of VFA's via the Na salts were mostly in the range 90–110%. Errors were presumably in measurement of samples on to the pyrophosphoric acid, by absorption of VFA's in the rubber tube, carry-over of traces of water, etc. Errors when free VFA's were applied directly to the chromatogram were considerably less. In both cases the errors in the relative proportions of the VFA's, the main interest of the present study, were smaller. α -Methylbutyric acid ('active valeric acid') was presumably measured with isovaleric acid, since the 4 ft. column used was not sufficient for its resolution. The commercial 'isovaleric acid' used for control purposes was presumably also a mixture of these acids (see James & Martin, 1952).

RESULTS

Feeding of sheep

The daily dietary regime for the various experiments is given in Table 1, and the protein and dry-matter contents of the feeding stuffs are given in Table 2. Unless otherwise stated, the animals had been on the same diet for at least a fortnight before the day of the experiment. Feeds were nearly always consumed within half an hour of being given.

Time course of evolution of ammonia and higher volatile fatty acids

In Exps. 1–6 the content of ammonia and of C_4 and (C_6 and higher) VFA's (determined by the procedure of Elsdén) was determined in rumen-liquor samples taken at different times, expressed as mg. equiv./100 ml. rumen liquor and plotted against time (Figs. 1–3). The results show a marked parallelism in the evolution of ammonia and of the VFA's, especially (C_6 and higher).

Recognition of presence of branched-chain C_6 volatile fatty acids

Figs. 4 and 5 show analyses, by the method of James & Martin, of typical rumen-liquor VFA samples before and after the addition, respectively,

Table 1. *Dietary regime of fistulated Cheviot ewes*

(Weights of food are air dry, as fed. Analytical and other data on the foods are given in Table 2.)

Exp. no.	Sheep no.	Time of feeding						Date
		07.00 hr.		11.00 hr.		16.00 hr.		
		Food	Wt. (g.)	Food	Wt. (g.)	Food	Wt. (g.)	
1	716	Hay I	240	Hay I	240	Hay I	240	5. viii. 49
2	717	Concentrate I Casein	150 50	Hay I	200	Hay I	200	5. viii. 49
3	718	Concentrate I	150	Hay I	200	Hay I	200	21. iii. 49
4	716	Concentrate I Casein	150 50	Hay I	200	Hay I	200	21. iii. 49
5	716	Fresh grass	800	Fresh grass	800	Fresh grass	800	24. v. 50
6	718	Fresh grass	800	Fresh grass	800	Fresh grass	800	24. v. 50
7	718	Concentrate II Casein	600 50	Hay II	300	Hay II	300	5. xii. 50
8	718	Concentrate II Casein	600 50	Hay II	300	Hay II	300	12. xii. 50
9	722	Concentrate II Casein	400 100	Hay II	300	Hay II	300	5. xii. 50
10*	719	Concentrate II Casein	400 100	Hay II	300	Hay II	300	12. xii. 50
11, 12	719	Frozen grass	1600	Frozen grass	1600	Frozen grass	1600	19. x. 50 and 31. x. 50
13, 14	722	Dried grass	360	Dried grass	360	Dried grass	360	19. x. 50 and 31. x. 50
15, 16	716	Silage	1455	Silage	1455	Silage	1455	19. x. 50 and 31. x. 50

* Only on this diet for 6 days previous to experiment.

Table 2. *Composition of feeding stuffs*
(see Table 1 and text)

Feeding stuff	N (Kjeldahl) (%)	Dry matter (%)	N as % of dry matter
Hay I*	1.35	87.8	1.54
Hay II*	1.37	89.2	1.54
Concentrate I†	2.61	85.7	3.04
Concentrate II‡	2.35	86.2	2.73
Frozen grass§	0.63	18.7	3.37
Dried grass	3.02	86.8	3.48
Silage¶	0.59	16.0	3.69
Casein**	12-13	85-90	—

* Mixed rye grass-clover hays from Aberdeenshire.

† 8 parts ground maize, 2 parts wheat bran, 2 parts crushed oats, 1 part linseed-cake meal and 1 part white-fish meal.

‡ 8 parts ground maize, 2 parts wheat bran and 2 parts crushed oats.

§ A grass-clover mixture cut at the Duthie-Craibstone Farm in May 1950 and stored at -20°.

|| The same grass-clover mixture dried commercially.

¶ The same grass-clover mixture chopped and ensiled in small-scale concrete silos without addition.

** Various preparations by commercial firms.

of commercial *n*-valeric and 'isovaleric' acids. Fig. 6, for comparison, shows an analysis of a made-up mixture of acetic, propionic, isobutyric, *n*-butyric, 'isovaleric', *n*-valeric, γ -methylvaleric

('isocaproic') and hexanoic acids. From these experiments the predominance of branched-chain C_5 acids and the low concentration of C_6 acids in

Fig. 1. Concentrations of ammonia and higher VFA's in rumen liquor of sheep after feeding concentrates (07.30 hr.) and hay (11.00 hr.) (Exp. 3, ----), and concentrates with casein (07.30 hr.) and hay (11.00 hr.) (Exp. 4, —).

rumen liquor is apparent. The forms of the curves leave no doubt as to the identities of the C_4 and lower VFA's.

Fig. 2. Concentrations of ammonia and higher VFA's in rumen liquor of sheep after feeding concentrates with casein (09.00 hr.) and hay (11.00 hr.) (Exp. 2, —), and hay only (09.00 hr. and 11.00 hr.) (Exp. 1, ----).

Fig. 3. Concentrations of ammonia and higher VFA's in rumen liquor of sheep after feeding fresh grass (07.00 and 11.00 hr.). (Exp. 5, —; Exp. 6, ----.)

Fig. 4. Gas-liquid partition chromatogram of VFA's from rumen liquor of sheep (a) without and (b) with addition of *n*-valeric acid.

Fig. 5. Gas-liquid partition chromatogram of VFA's from rumen liquor of sheep (a) without and (b) with addition of commercial 'isovaleric' acid.

Fig. 6. Gas-liquid partition chromatogram of a mixture, in roughly equal proportions, of commercial acetic, propionic, isobutyric, n-butyric, 'isovaleric', n-valeric, γ -methylvaleric ('isocaproic') and hexanoic acids.

Analyses of changes of volatile fatty acid composition of rumen liquor on feeding

In Exps. 7–16 the evolution of ammonia was followed during the day (Chalmers & Syngé, to be published). Curves for this are given in Figs. 7 and 8. Larger samples of rumen liquor were taken for VFA

analysis by the method of James & Martin at 7 a.m. immediately before feeding and at times (indicated by the arrows on the ammonia curves) when it was hoped that the ammonia concentration would be high. The results of these analyses are set out in Table 3. The large absolute and relative increases of the branched-chain isomers when casein

Fig. 7. Time course of ammonia concentration in rumen liquor of sheep fed casein and concentrate at 07.00 hr. and hay at 11.00 hr. Samples for analysis of VFA's were taken before feeding and at the times indicated by the arrows on the curves.

Fig. 8. Time course of ammonia concentration in rumen liquor of sheep fed (at 07.00 and 11.00 hr.) frozen grass (— · — · —), dried grass (----) and silage (—). Samples for analysis of VFA's were taken before feeding and at the times indicated by the arrows on the curves.

Table 3. *Changes in concentrations of volatile fatty acids in rumen liquor of sheep on different diets*
(For details of animals, feeding, times of sampling, and concurrent ammonia concentrations see Tables 1 and 2, Figs. 7 and 8 and text.)

Exp. no.	Feed		Total VFA's (mg. equiv./ 100 ml.)	Individual VFA's as % of total VFA (molecular basis)						
				Acetic	Propionic	iso- Butyric	n- Butyric	Branched C ₅	n- Valeric	C ₆ etc.
7	Concentrate + 50 g. casein	Before	5.6	64.4	18.0	2.8	7.7	1.2	0.9	Trace
		After	11.8	56.9	23.7	1.5	8.5	3.9	1.4	Trace
8	Concentrate + 50 g. casein	Before	7.3	65.5	22.9	1.4	10.6	2.7	1.0	Trace
		After	14.4	45.5	23.5	2.0	10.6	3.5	2.3	Trace
9	Concentrate + 100 g. casein	Before	8.6	60.0	14.3	1.3	10.2	2.1	1.0	0.8
		After	10.6	67.0	18.5	2.5	11.7	5.0	2.4	1.9
10	Concentrate + 100 g. casein	Before	10.6	55.7	20.6	1.7	19.3	3.1	2.3	Trace
		After	16.0	53.1	21.7	2.7	16.6	3.6	3.3	Trace
11	Frozen grass	Before	8.6	65.7	17.1	2.1	9.3	3.1	1.3	0.4
		After	11.6	54.0	22.6	1.7	14.2	1.1	1.2	Trace
12	Frozen grass	Before	5.8	67.2	17.1	2.2	9.3	2.9	1.6	Trace
		After	13.8	55.2	24.3	1.2	12.5	1.3	1.6	Trace
13	Dried grass	Before	9.8	69.5	18.6	1.4	8.1	1.9	0.7	Trace
		After	14.9	57.7	22.2	0.8	8.4	1.4	0.8	Trace
14	Dried grass	Before	9.4	55.5	15.7	1.0	7.6	1.3	1.1	Trace
		After	13.5	63.5	26.0	0.5	11.9	0.8	1.5	Trace
15	Silage	Before	5.5	70.0	16.2	2.6	8.6	3.2	0.6	0.9
		After	11.0	53.2	25.4	1.3	7.4	2.5	1.7	0.8
16	Silage	Before	7.5	72.6	14.9	1.6	7.7	1.9	1.0	Trace
		After	14.5	66.6	23.6	1.5	10.7	2.7	2.3	Trace
Analysis of silage fed in Exps. 15 and 16			10.6	74.0	10.7	Nil	5.1	Nil	1.1	Nil

was fed are apparent. With the grass products there is a relative fall and little absolute increase in the concentrations of branched-chain VFA's, except on feeding silage where there is a substantial absolute increase. This may be correlated with the higher concentrations of ammonia found on feeding the silage (Fig. 8). The parallel analysis of the VFA's of the silage fed (Table 3, last entry; cf. Brouwer & Nijkamp, 1950) shows that little or no preformed branched-chain VFA's were present. They have, however, been reported in certain silages by Brahm (1925, 1927).

DISCUSSION

It is assumed that $\alpha\alpha$ -dimethylpropionic (trimethyl-acetic, pivalic) acid, which has never so far been found in nature, is absent from rumen contents. This is not readily separated from *n*-butyric acid by the methods used. It follows that *isobutyric*, *n*-butyric, '*isovaleric*' (α -methylbutyric and/or *isovaleric*) and *n*-valeric acids occurred in the rumen contents of the sheep studied; C_6 homologues, if present, occurred only in traces. It has for some time been recognized that volatile fatty acids other than acetic, propionic and *n*-butyric acids are present (cf. especially Elsdén, 1946), but the demonstration of their nature has now been made simple by use of the technique of James & Martin (1952).

Since Elsdén & Phillipson (1948) reviewed knowledge of these acids in rumen contents, Schambye & Phillipson (1949) determined 'butyric plus higher' acids, and Reid (1950) found in addition to acetic 'small amounts of propionic, butyric and at least one other acid' in the systemic blood of the sheep. A more detailed study has been made by McClymont (1951) who determined higher VFA's in the rumen of oxen fed on various diets. C_4 isomers ranged from 10.4 to 18.1 % of total VFA's and (C_5 and higher) from 2.6 to 5.1 % (molecular basis). In a special study of the latter fraction (diet not specified) C_5 isomers accounted for 51 % and C_6 for 38 %. Since the present work was done Gray, Pilgrim, Rodda & Weller (1951) have published a preliminary account of a detailed study of the VFA's from sheep rumen contents. The main fractionation was by unspecified partition-chromatographic procedures. A sheep fed wheaten hay gave *n*-butyric acid, 9.0; *isobutyric* acid, 0.7; C_5 acids, 2.6; C_6 acids, 0.7 %; C_7 (?), trace. It is not clear whether these percentages are on a molecular or weight basis, but their figure for *isobutyric* acid, determined by 'a separate method', is somewhat lower in relation to *n*-butyric acid than was usually found in the present work. Gray *et al.* (1951) also found by comparing *R* values that a greater proportion of the C_5 acids were *n*-valeric than were branched-chain acids. Since this is the

opposite of what was usually found here, and since comparing *R* values is a much less reliable method of identification than the running of mixed chromatograms with natural and synthetic materials, a decision must await the publication by Gray and colleagues of fuller data.

Discussion of the origin and fate of these acids of the rumen is deferred to the following paper, where these problems are considered in relation also to the *in vitro* data there recorded. Although branched-chain C_4 and C_5 VFA's are well known constituents of certain plants and have been widely recognized as fermentation and putrefaction products derived from proteins by the action of micro-organisms, there have been surprisingly few observations of their occurrence in animals, except in intestinal worms (Flury, 1912; Epps, Weiner & Bueding, 1950) and, esterified, in the fat of porpoises and whales (e.g. Lovern, 1934). Brieger (1877) obtained evidence for the occurrence of *isobutyric* acid (as well as acetic and *n*-butyric acids) in human faeces, and Kline (1935) found it in human urine. The present work establishes that, in the rumen of the sheep, a significant proportion of the C_4 VFA's and the greater part of the C_5 VFA's have branched-chain structures and demonstrates a parallelism in the changes of concentrations of ammonia and of the C_4 and C_5 acids in the rumen after administration of protein-rich foods. The only easy interpretation of these facts seems to be that suggested above, namely that a principal source of both the ammonia and the acids is deaminative attack by micro-organisms on the amino-acids of the protein fed.

SUMMARY

1. Using the gas-liquid partition chromatogram of James & Martin (1952) it was found that branched-chain lower fatty acids occur in the rumen of the sheep on a variety of diets. A significant proportion of the C_4 acids was *isobutyric* acid, and branched-chain isomers often made up the greater part of the C_5 acids. C_6 acids were present only in small amounts.
2. The concentrations of ammonia and of the various volatile fatty acids in the rumen were determined at different times after feeding.
3. Increased ammonia concentration was correlated with increased concentrations of *isobutyric* acid and of the C_5 acids, which are therefore considered to arise from microbial attack on protein in the rumen.

I am very grateful to Dr A. T. James and Dr A. J. P. Martin, F.R.S., for instruction in the use of their gas-liquid partition chromatogram, and to Mr R. S. Reid and Mr G. Masson for constructing the necessary apparatus.

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 * (Note added in proof.) See McDonald, I. W. (1952). *Biochem. J.* **51**, 86.

Degradation of Protein in the Rumen of the Sheep

2. THE ACTION OF RUMEN MICRO-ORGANISMS ON AMINO-ACIDS

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In the previous paper (el-Shazly, 1952) evidence that ammonia is formed from protein in the rumen was reviewed and it was suggested that some of the volatile fatty acids (VFA's) found in the rumen originate mainly from the breakdown of protein. To investigate more closely the character of this breakdown the action of rumen micro-organisms on free amino-acids has now been studied. Only low concentrations of free amino-acids exist in the rumen liquor, but there is evidence (Sym, 1938) of protease activity by the micro-organisms; McDonald (1948) has measured the disappearance of zein from the rumen and Chalmers & Synge (to be published) have observed the dissolution of lumps of denatured casein. The nature of this proteolytic action, which is likely to prove of great importance for the protein economy of the animal, must await more detailed study. The present work has been concerned solely with the fate of the free amino-acids which it is reasonable to assume are liberated in the proteolysis.

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The best course would have been to study the fate of amino-acids added in low concentrations to whole rumen liquor within a very short time of its removal from the animal, but this was not technically feasible. It was, however, possible to observe the disappearance of somewhat high concentrations of amino-acids added to freshly removed rumen liquor during anaerobic incubation for a few hours; the high concentrations of ammonia and VFA's already present prevented detailed study of new formation of these products from the added amino-acids. However, following recommendations of Dr S. R. Elsdon, conditions were found for using washed suspensions of mixed rumen micro-organisms which attacked amino-acids present in mixtures at much the same relative rates as these were attacked in whole rumen liquor. Under these circumstances it was relatively easy to determine the end products of the fermentation and to construct a rough balance sheet for the reactions. Although the reactions may take a different course under physiological conditions (where the amino-acid concentrations are lower, other essential nutrients for the micro-