not appreciably affected by temperature. Most of the material given was eliminated in the first few days after dosing, but small amounts continued to be excreted for several weeks. Even with the much smaller quantities eliminated on these later days, about half was still unconjugated.

The reduction of the nitro group in *p*-nitrobenzoic acid which was observed indicates the presence of another possible detoxication mechanism in locusts, since reduction of nitro groups often lowers toxicity (cf. Smith *et al.* 1953). This reduction is not unexpected since Dennel (1949) has observed that insect blood has a low redox potential.

The formation of hippuric acids in the locust is interesting in connexion with the comparative biochemistry of nitrogen metabolism. Locusts differ from the common experimental animals in that uric acid is a major end-product of their nitrogen metabolism (Chauvin, 1941). The hen, which also eliminates most of its nitrogen as uric acid, uses ornithine for the detoxication of aromatic acids, and it is sometimes suggested (Quick, 1948; Baldwin, 1948) that other animals with uricotelic metabolism, such as reptiles, may also form ornithuric acids. The only reptile which appears to have been studied is a turtle, which produced hippuric acids (Komori & Sendyu, 1926), but it was not stated whether this turtle had a ureotelic or uricotelic metabolism (cf. Baldwin, 1952). The present work suggests that there may be no general connexion between uric acid production and the use of ornithine as a conjugating agent.

SUMMARY

1. The metabolism of benzoic, *p*-nitrobenzoic, *p*-aminobenzoic and salicylic acids has been studied in the adult locust.

2. The glycine conjugates of these acids have been identified in the excreta by spectroscopic and paper chromatographic techniques.

3. Quantitative measurements showed that about 20% of *p*-aminobenzoic acid was converted into *p*-aminohippuric acid.

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Some Observations on Volatile Fatty Acids in the Sheep's Rumen

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A number of investigations of the volatile fatty acids (VFA's) of the rumen have been reported since the subject was reviewed by Elsden & Phillipson (1948). Particular interest has focused on rumen VFA's other than acetic, propionic and butyric acids. McClymont (1951) made a detailed study of the higher VFA's in the rumen of an ox fed various diets, and Gray, Pilgrim, Rodda & Weller (1952) used a combination of liquid/liquid partition chromatography and chemical methods to demonstrate the presence of formic, *iso*butyric and hexanoic acids, a valeric acid isomer and possibly heptanoic acid in the sheep rumen. The analysis of mixtures of VFA's has been greatly simplified, however, by the development of the method of James & Martin (1952) using gas/liquid partition

chromatography. Shazly (1952*a*) used this method to demonstrate unequivocally that *iso*butyric acid and one or more branched-chain valeric acid isomers occur in the rumen of sheep fed various diets. The production of acetate, propionate, butyrate, valerate and hexanoate by a Gram-negative coccus isolated from the rumen of the sheep has been reported by Elsden & Lewis (1953). Gray *et al.* (1952) have used acids labelled with ^{14}C to show that valeric acid may be synthesized in the rumen from acetic and propionic acids.

The work of Shazly (1952*a*, *b*) relating protein degradation in the sheep rumen to branched-chain VFA production has been extended in the present studies, which were undertaken at the suggestion of Dr R. L. M. Synge. In the first part of the work, which was carried out at the Rowett Research Institute, complete analyses were made of the VFA's produced in the rumen of sheep fed various high-protein diets. Chromatographic evidence was obtained of the presence of *iso*valeric and 2-methylbutyric acids, the two naturally occurring branched-chain valeric acid isomers, but VFA's higher than C_5 were not observed. The investigations were completed at the A.R.C. Institute of Animal Physiology. A partially purified sample of dextrorotatory 2-methylbutyric acid was isolated from the rumen contents of sheep fed a casein-rich diet.

MATERIALS AND METHODS

The experiments at the Rowett Research Institute were carried out using Cheviot ewes fitted with permanent rumen fistulae. In later work, at Babraham, Clun Forest sheep with rumen fistulae were employed.

Volatile fatty acids. Racemic 2-methylbutyric acid and *iso*valeric acid were synthesized by carbonation of the Grignard complexes of *sec.*-butyl and *iso*butyl chlorides respectively, as described by Gilman & Kirby (1932) for 2-methylbutyric acid. Other VFA's used for control purposes were 'laboratory reagent' quality (British Drug Houses Ltd.) and were redistilled before use. Each acid had the calculated equivalent weight on titration with alkali, and was homogeneous when examined by the chromatographic methods mentioned below. The 2-methylbutyric acid was partially resolved by fractional crystallization of the brucine salt (Schutz & Marckwald, 1896); final preparations showed the following specific rotations (measured in 1 dm. tube):

$$[\alpha]_D^{22} = +12{\cdot}9 \pm 0{\cdot}01°: [\alpha]_D^{22} = -6{\cdot}7 \pm 0{\cdot}01°.$$

The reported value of the dextrorotatory form is

$$[\alpha]_D^{20} = +17{\cdot}85°$$

(Marckwald, 1899).

Analysis of volatile fatty acids. The gas/liquid partition-chromatography method of James & Martin (1952) was employed at a temperature of 135° using methyl cellosolve in the heating jacket. Satisfactory separations of the branched-chain valeric acid isomers were obtained by using an 11 ft. column of modified composition. The amount of stearic acid in the liquid phase (DC 550 silicone, Albright and Wilson Ltd., Oldbury, Birmingham) was increased from 10 to 15% (w/w). A nitrogen flow rate of 15 ml./min. was used. All percentages of VFA's are expressed on a molecular basis.

Determination of total VFA's in rumen liquor. The general procedure of McAnally (1944) was followed. Rumen contents were strained through muslin, deproteinized with an equal volume of N-H_2SO_4 saturated with $MgSO_4$, $7H_2O$ and filtered; 2–5 ml. of the filtrate were distilled in a Markham still (Markham, 1942). Three 50 ml. portions of distillate were collected, and titrated with 0·02 N-NaOH under CO_2-free conditions, using phenolphthalein as indicator. When large samples of rumen VFA's were required for fractionation by liquid/gel partition chromatography, the filtrate (50 ml.) was steam distilled and 1–1·5 l. of distillate were collected.

Preliminary fractionation of rumen VFA's. Since only relatively small amounts of C_4 and C_5 VFA's are normally present in sheep rumen liquor, a preliminary concentration of the higher acids was effected by liquid/gel partition chromatography, using the method of Elsden (1946). Samples containing about 1 m-mole of VFA were fractionated on 40 g. silica-gel columns. A fresh column was used for each sample. The bands containing the C_4 and C_5 acids were collected together and titrated with 0·02 N-NaOH under CO_2-free conditions; the salts of the acids were retained for analysis by gas/liquid partition chromatography. Collection of eluate was commenced early in the development of the chromatogram to ensure that any fast-moving bands of higher VFA's present in amounts too small to be observed visually were collected with the C_4 and C_5 acids. The propionic acid band was also collected and titrated, and the acetic acid content of the mixture determined by difference. Formic acid, if present, is not distinguished from acetic acid by this method.

Table 1. *Diets of fistulated Cheviot ewes*

Air-dried materials/24 hr.	g. N/day (approx.)
1. 300 g. groundnut meal, 300 g. ground maize, 300 g. hay	24
2. 100 g. casein, 300 g. ground maize, 300 g. hay	21
3. 750 g. ground maize, 300 g. hay	15
4. 600 g. hay	7

RESULTS

VFA analysis of rumen contents of sheep receiving protein-rich rations

Four ewes were maintained on the diets shown in Table 1. The sheep received half of the daily ration at 12 hr. intervals (8.0 a.m. and 8.0 p.m.) and were trained to eat the food within 1 hr. It was planned to feed the different rations to each sheep in turn, but one of them (sheep 722) was lost after the first part of the experiment. The control diet of hay was therefore eliminated and for the remainder of the experiment only three sheep on the high-protein

Table 2. *Complete analysis of rumen volatile fatty acids (VFA's)*

Date	Sheep	Diet (Table 1)	Time after feeding (hr.)	Total VFA concentration (m-moles/l.)	Molecular percentages of individual VFA's						
					Acetic	Propionic	*iso*Butyric	Butyric	*iso*Valeric	2-Methyl-butyric	Valeric
19. xii. 51	43	Groundnut	2	184	60	26	0·9	10·1	0·9	0·5	1·6
			5	142	44	32	1·9	17·4	1·4	0·7	2·6
			8	97	41	34	2·4	19·0	0·9	0·9	1·8
	716	Maize	2	89	46	32	0·5	18·0	1·3	0·4	1·8
			5	89	56	28	0·4	13·3	1·0	0·3	1·0
			8	104	56	32	0·5	8·9	0·9	0·4	1·3
	718	Casein	2	77	60	22	2·5	7·9	2·8	1·7	3·1
			5	87	70	18	1·3	7·3	1·1	1·0	1·3
			8	92	68	18	1·4	9·0	1·4	1·0	1·2
	722	Hay	2	117	81	13	0·6	4·3	0·3	0·3	0·5
			5	113	77	19	0·5	2·4	0·3	0·3	0·5
			8	120	78	18	0·4	2·9	0·2	0·2	0·3
24. i. 52	43	Maize	2	102	52	35	0·2	12·0	0·3	0·2	0·3
			5	102	50	24	0·5	20·0	0·7	0·2	4·6
			8	94	48	18	0·5	28·9	0·7	0·1	3·8
	716	Casein	2	103	51	25	3·8	10·3	3·6	2·2	4·1
			5	97	58	21	3·1	10·8	2·7	2·4	2·0
			8	95	57	24	2·8	10·3	1·9	1·7	2·3
	718	Groundnut	2	111	43	26	2·2	21·6	2·2	1·9	3·1
			5	122	45	26	2·7	18·8	2·7	1·6	3·2
			8	103	37	28	3·5	24·1	2·4	1·5	3·5
21. ii. 52	43	Casein	2	131	53	24	3·5	13·0	1·9	2·1	2·5
			5	132	48	21	3·2	19·8	2·7	2·4	2·9
			8	113	53	26	2·4	12·0	1·9	1·6	3·1
	716	Groundnut	2	146	48	23	1·9	21·9	1·5	1·1	2·6
			5	143	51	27	2·3	14·4	1·9	1·5	1·9
			8	119	49	29	2·0	16·1	0·9	0·8	2·2
	718	Maize	2	116	—	—	—	—	—	—	—
			5	122	41	30	3·3	19·0	1·8	1·2	3·7
			8	94	—	—	—	—	—	—	—

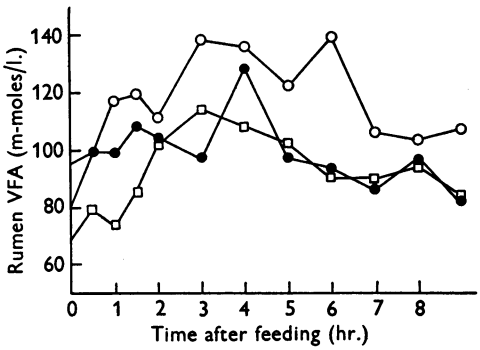

Fig. 1. Changes in rumen VFA concn. (m-moles/l.) after feeding of sheep maintained on diets rich in casein, ●—●; groundnut, ○—○; and flaked maize, □—□.

rations were employed. When the diets were changed the animals were not sampled for at least 1 month, to allow adaptation to the new diets. Rumen samples for complete VFA analysis were taken at 2, 5 and 8 hr. intervals after feeding, and the analytical results are shown in Table 2. As a background to these analyses, smaller samples for determination of total rumen-VFA's were taken at short intervals throughout the same day. The results of a typical experiment are shown in Fig. 1.

Since the procedures described above for the complete analysis of rumen VFA's do not distinguish formic from acetic acid, a series of samples of rumen VFA's from the same sheep were examined by gas/liquid chromatography without preliminary liquid/gel partition. Traces of formic acid were occasionally observed, but in no instance did this exceed 1% of the total VFA's.

Partial purification of the 2-methylbutyric acid of rumen contents

The figures for 2-methylbutyric acid shown in Table 2 are based on the size of the zone observed when the rumen VFA's were examined by gas/liquid chromatography. The chromatographic evidence for the occurrence of 2-methylbutyric acid in rumen contents is summarized below.

Addition of synthetic 2-methylbutyric acid to a sample of rumen VFA's suspected to contain this acid did not increase the number of bands obtained on the gas/liquid chromatogram, but only enlarged the '2-methylbutyric acid' band. In addition, a sample of rumen VFA's was examined on the gas/liquid chromatogram (11 ft. column) and the titration cell contents were changed after the emergence of the *iso*valeric acid band. The next band was collected and recovered from the cell contents by steam distillation. This acid was again examined chromatographically, alone and mixed with synthetic 2-methylbutyric acid. In each case only one band was observed, which emerged after a time which had been established by control experiments to be characteristic for 2-methylbutyric acid under the operating conditions employed.

The separation of 2-methylbutyric acid from the other naturally occurring valeric acid isomers present in rumen contents by techniques other than gas/liquid chromatography has not been reported, although the two branched-chain valeric acids can be separated together as a mixture from valeric acid by the liquid/gel partition method of Moyle, Baldwin & Scarisbrick (1948). Since gas/liquid chromatography has not yet been developed to operate on a preparative scale, further evidence for the occurrence of 2-methylbutyric acid ('active' valeric acid) in rumen contents has been obtained by examining the optical activity of a partially purified sample. The higher VFA's from a large sample were concentrated by successively partitioning the VFA mixture between ether and water at pH 4·5. The 'active' valeric acid content of the resulting acid mixture was compared with the optical activity of the solution.

A sample of rumen contents (700–800 ml.) was taken 4 hr. after feeding from a sheep receiving a casein-rich diet (1 kg. hay (N, 1·3%) and 150 g. casein/day), strained through muslin and treated with an equal vol. of N-H_2SO_4 saturated with $MgSO_4$, $7H_2O$. After centrifuging (2000 *g*, for about 20 min.) the supernatant (1350 ml.) was extracted with ether in a continuous extraction apparatus (Vogel, 1951) until the VFA content of the aqueous layer was reduced to about 5% of its original value. The VFA's in the ether were then removed by shaking with a slight excess of alkali. This procedure was repeated 3 days later with a further 700 ml. of rumen contents obtained from the same sheep. The extracted VFA's were combined, the total VFA was determined (125 m-moles) and a sample was analysed chromatographically. Acetic (60%), propionic (17%), *iso*butyric (4%), butyric (10%), *iso*valeric (approx. 3%), 2-methylbutyric (approx. 2%) and valeric acid (approx. 4%) were present. An aqueous solution of the extracted VFA's was adjusted to pH 4·5 with 50% (w/v) aqueous H_3PO_4, diluted to 250 ml. and shaken with an equal volume of water-saturated ether. The ether layer was then separated, extracted with a slight excess of N-NaOH, and the VFA fraction so obtained was partitioned between ether and water at pH 4·5 as before. The aqueous layers were rejected at each stage and the course of the higher-VFA separation was followed by analysis. This procedure was repeated six times, when 16 m-moles of VFA remained. At this stage, an effort was made to remove any optically active non-acidic substances that may have been present by steam distillation under alkaline conditions. The solution was then acidified and the acids were steam distilled. After neutralization with NaOH, the distillate was concentrated to a small volume, the pH adjusted to 7·0 with 2 N-H_2SO_4 and the solution made up to 20 ml. The VFA content of the solution was determined (14·1 m-moles), and a sample was analysed for VFA's. *Iso*butyric (10%), butyric (13%), *iso*valeric (28%), 2-methylbutyric (13%) and valeric acids (36%) were present. The 2-methylbutyric acid content of the solution was thus 0·93% (w/v). Polarimetric observation of the solution in a 4 dm.

tube showed a rotation of +0·39°, or $[\alpha]_D^{22} = +10{\cdot}5 \pm 0{\cdot}3°$ (as Na salt but calculated for free acids; *c*, 0·93) assuming that only 2-methylbutyric acid contributed to the optical activity. A solution of the sodium salt (pH 7·0) of partially resolved dextrorotatory 2-methylbutyric acid

$$([\alpha]_D^{22} = +12{\cdot}9°)$$

was prepared at a concentration of 1·29 %, i.e. equivalent in optical rotatory power to the solution containing isolated 2-methylbutyric acid, assuming that (*a*) the specific rotation of the dextrorotatory acid is $[\alpha]_D^{20} = +17{\cdot}85°$ (Marckwald, 1899), and (*b*) that the optical activity of the isolated VFA solution is due only to dextrorotatory 2-methylbutyric acid. The artificial solution contained in addition the other acids present in the isolated sample at similar concentrations in order to examine their possible effect on the optical activity of the 2-methylbutyric acid. Polarimetric observation of the solution in a 4 dm. tube gave a rotation of +0·38°, or $[\alpha]_D^{22} + 7{\cdot}4 \pm 0{\cdot}3°$ (same basis as for natural material). The material in rumen liquor, if isolated, would on this basis have $[\alpha]_D^{22} = \frac{10{\cdot}5 \times 12{\cdot}9}{7{\cdot}4} = +18{\cdot}3°$, in good agreement with the value recorded by Marckwald (1899).

Rumen VFA's of fasted sheep

Rumen samples for VFA analysis (without preliminary liquid/gel fractionation) were taken from two sheep receiving diets rich in casein (150 g. casein, 1 kg. hay (N, 1·3 %)/day) and dried grass (600 g. dried grass (N, 2·6 %), 200 g. hay (N, 1·3 %)/day), respectively, over a 48 hr. period after feeding. The total VFA level fell steadily (Fig. 2) but the concentration of the branched-chain VFA's (*iso*butyric, *iso*valeric and 2-methylbutyric acids) showed little change. Formic acid was absent from all samples, but traces of C_6 acids, tentatively identified as γ-methyvaleric and hexanoic acids by the times at which they emerged from the gas/liquid partition column, were observed in the later samples.

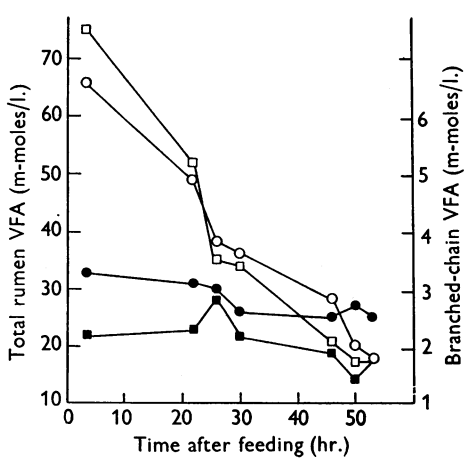

Fig. 2. Changes in total and branched-chain VFA concn. (m-moles/l.) in the rumen of fasted sheep maintained on diets rich in casein (total VFA's, ○—○, branched-chain VFA's, ●—●) and dried grass (total VFA's, □—□, branched-chain VFA's, ■—■).

DISCUSSION

The occurrence of dextrorotatory 2-methylbutyric acid in the sheep rumen has been established. Measurable quantities of *iso*butyric acid and the three naturally occurring valeric acid isomers were observed in each sample of rumen contents examined (Table 2). Some correlation between the quantity of protein fed and the amount of branched-chain VFA's produced can be seen; this aspect of protein degradation in the rumen has been examined more fully by Shazly (1952*a*). The comparatively large amounts of valeric acid observed, particularly on a maize diet, support the findings of Gray *et al.* (1952) on the production of valeric acid in the rumen. The absence of C_6 and C_7 VFA's in rumen samples taken from sheep under normal conditions was unexpected in view of the findings of McClymont (1951), who observed a C_6 acid in the ox rumen, and Gray *et al.* (1952), who reported the occurrence of C_6 and C_7 acids in the sheep rumen. Traces of C_6 acids were found in the rumen of fasted sheep. Small amounts of formic acid (1 % of the total VFA's) were infrequently observed in rumen samples, in contrast to the observations of Gray *et al.* (1952) who reported 0–5 % formate in the rumen VFA's of sheep fed wheaten hay or lucerne hay.

The amounts of branched-chain VFA's in the rumen almost certainly depends on the rate and extent of degradation of ingested and microbial protein and on the rate of absorption of the acids produced. If the latter factor is fairly constant, the relatively slight changes in rumen branched-chain VFA level observed on fasting probably indicates increased microbial degradation which compensates for the steadily decreasing amounts of ingested protein present in the rumen.

The production of ammonia in the rumen as a result of protein degradation was demonstrated by McDonald (1948, 1952). Shazly (1952*a*) measured ammonia and VFA production in the rumen of sheep fed various diets, and ruminal ammonia levels in sheep maintained on the diets shown in Table 1 have been reported (Annison, Chalmers, Marshall & Synge, 1954). The nutritional significance of the extent to which ingested protein is degraded in the rumen has been discussed by Chalmers, Cuthbertson & Synge (1954), Chalmers & Synge (1954) and Annison *et al.* (1954).

Recent investigations have indicated that branched-chain fatty acids are probably more widely distributed in animal tissues than was once considered likely (cf. Shazly, 1952*a*). Branched-chain acids have been revealed as minor constituents

of wool grease (Weitkamp, 1945), butterfat (Hansen & Shorland, 1951, 1952; Hansen, Shorland & Cooke, 1951), ox fat (Hansen, Shorland & Cooke, 1952*a*) and mutton fat (Hansen, Shorland & Cooke, 1952*b*, 1953), and substantial quantities have been found in the body fat of whales and porpoises (Lovern, 1934). Optically active 4-methylhexanoic acid (contaminated with traces of 2-methylhexanoic acid) has been isolated from the coccygeal glands of ducks, together with appreciable amounts of other unidentified alkyl-substituted acids (Weitzel & Lennert, 1951). Brouwer & Nijkamp (1953) have reported the occurrence of 2-methylbutyric and *iso*valeric acids in the hair grease of dogs.

The stereochemistry of the C_{15}, C_{17} and C_{19} *ante-iso* acids isolated from mutton fat (Hansen *et al.* 1952*b*, 1953) and wool fat (Weitkamp, 1945) has been discussed by Klyne (1953), who showed that these acids are almost certainly L-methyl alkanoic acids. Dextrorotatory 2-methylbutyric acid is also a (+)*ante-iso* acid or L-methyl alkanoic acid (Klyne, 1953) and would arise by degradation of L-isoleucine. This supports the postulated origin of these VFA's from protein in the rumen and lends support to the suggestion of Shazly (1952*b*) that the branched-chain VFA's present in the rumen might possibly be precursors of the higher branched-chain acids found in animal fats by Hansen and his co-workers.

SUMMARY

1. The presence of *iso*butyric, *iso*valeric and 2-methylbutyric acids in the rumen of sheep maintained on various diets has been demonstrated.

2. Polarimetric observation of a partially purified sample of 2-methylbutyric acid isolated from the rumen contents of a sheep fed a casein-rich diet revealed that only the dextrorotatory form was present.

3. Volatile fatty acids (VFA's) higher than C_5 have not been observed in the sheep's rumen under normal conditions, although traces of C_6 acid have been found in the rumen of fasted sheep. Traces of formic acid were occasionally observed.

4. The changes in the VFA content of the rumen of sheep when fasted have been examined. Although the total VFA level fell steadily, the concentration of the branched-chain VFA's showed only a slight decrease.

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