Urinary aromatic acid excretion by fed and fasted sheep in relation to protein metabolism in the rumen

By A. K. MARTIN

Hannah Research Institute, Ayr KA6 5HL

(*Received* 10 *October* 1972 – *Accepted* 14 *February* 1973)

1. Two adult wether sheep were maintained on a diet of hay and two on a diet of dried grass for 3 weeks before starvation for a period of 10 d. Urinary excretion of the following acids was determined when the animals were fed and when they were fasted: total diethyl ether-soluble acids of hydrolysed and unhydrolysed urine, hippuric acid, benzoic acid and phenylacetic acid. By the 5th day of fasting, urinary output of all acids had attained stable levels that did not change during the remaining starvation period. The output of all urine fractions except phenylacetic acid declined rapidly during the first 4 d of fasting: phenylacetic acid output by all sheep increased to a maximum during the first 4 d of fast and then declined to stable values (0·42–0·73 g/24 h) which were greater than those observed when the sheep were fed. It is concluded that prolonged retention of food and microbial residues in the digestive tract is responsible for the large ouptut of phenylacetic acid in the urine of fasted sheep.

2. Solutions of casein which supplied between 6·3 and 26·5 g nitrogen/24 h were infused into the rumens (fifteen experiments) or abomasums (sixteen experiments) of eight adult wether sheep. Ruminal infusions of casein caused increments in the urinary excretion of diethyl ether-soluble acids and phenylacetic acid. Both these increments were described by linear regression equations ($P < 0{\cdot}001$), the coefficients of which showed that 284 ± 44 and 220 ± 21 mg benzoic acid equivalent were excreted as diethyl ether-soluble acids and phenylacetic acid respectively per g casein N infused. The phenylacetic acid excreted was equivalent to 95 % of the phenylalanine of the infused casein. No increments in urinary benzoic acid were observed. One sheep scoured when it was given an abomasal infusion of casein. This was the only animal to show any increment in urinary aromatic acids when casein was infused into the abomasum.

3. When four sheep were given two rations containing an excess of carbohydrate as sugar-beet pulp or rolled barley, 11 and 16 % respectively of their phenylalanine intakes were excreted in the urine as phenylacetic acid. When the same sheep were given two rations containing an excess of N as linseed meal or field beans, 51 and 59 % respectively of their phenylalanine intakes appeared in the urine as phenylacetic acid.

4. Methods for the determination of creatinine, and of benzoic, phenylacetic, 3-phenylpropionic, cinnamic, hippuric and phenaceturic acids are described.

5. It is suggested that the amount of phenylacetic acid excreted in the urine is a measure of the equilibrium occurring in the rumen between catabolism of phenylalanine and reutilization of the products of catabolism for phenylalanine synthesis.

The urinary aromatic acid excretion of sheep has been shown to vary widely with the amount and type of food eaten; between 2·5 and 16·5 g/24 h of benzoic acid, and 0·1 and 2·4 g/24 h of phenylacetic acid may be excreted together in some samples, with traces of 3-phenylpropionic and cinnamic acids (Martin, 1970 and unpublished observations). Urinary benzoic acid is almost entirely derived from precursors present in the diet: withholding food from sheep for 4 d has been found to reduce benzoic acid excretion to 0·2 g/24 h (Martin, 1969*b*), whereas urinary phenylacetic acid output remained unchanged from that observed when the sheep were given hay, i.e. 0·48 g/24 h.

Phenylacetic acid excretion in the urine of normal adult man (of similar body-weight to sheep) has been reported to vary between 0·04 and 0·37 g/24 h (Seakins,

1971; Van der Heiden, Wauters, Ketting, Duran & Wadman, 1971). However, disorders of intestinal absorption in man, such as cystic fibrosis and coeliac disease, result in greatly increased urinary outputs of phenylacetic acid. It has been shown that the microbial fermentation of phenylalanine in the large intestine is the source of the large amounts of phenylacetic acid excreted by these subjects (Seakins, 1971; Van der Heiden, Wadman, Ketting & De Bree, 1971; Van der Heiden *et al.* 1971). Earlier work with ruminants had shown that phenylalanine was fermented in the rumen of cattle (Patton & Kessler, 1967) and of sheep (Scott, Ward & Dawson, 1964) to yield phenylacetic acid.

The experiments to be described in this paper were designed to answer three questions. First, was the rather large (0·48 g/24 h) urinary output of phenylacetic acid observed after sheep were fasted for 4 d (Martin, 1969*b*) due to metabolism occurring in the body tissues or to metabolism in the rumen of food and microbial residues? Second, is the production of phenylacetic acid as an end-product of microbial fermentation of phenylalanine in sheep confined to the rumen or does it occur in other parts of the digestive tract? Third, how does the nature of the food given to sheep affect the degradation of dietary phenylalanine to phenylacetic acid? Analytical methods developed for these studies are also described.

EXPERIMENTAL

Animals

Wether sheep of four breeds were used: no. 1 was a 6-year-old Welsh; no. 2 was a 7-year-old Kent; no. 3 was a 7-year-old Hampshire and nos 4–14 inclusive were Suffolk Crosses of between 2 and 4 years of age. Sheep nos 1–5, 7, 9 and 11 had rumen cannulas and sheep nos 1, 3–5, 7, 9 and 12–14 had abomasal cannulas.

Urine and faeces collection

Sheep were harnessed for the collection of faeces and urine by the method described by Wainman & Paterson (1963). Faeces and urine were weighed and sampled for analysis daily. Samples were stored at -10° until required for analysis. During 24 h collections urine pH was maintained between 3 and 4 by the addition of sulphuric or hydrochloric acid to the urine reservoir at the start and on one or two occasions during the collection period. Addition of large amounts of acid at the start of the collection period was avoided as this resulted in the crystallization of hippuric and phenaceturic acids from concentrated urine samples.

Plan of experiments

Experiment 1

The rations described in Table 1 were given to sheep nos 1–4 for 3 weeks. At the start of the 3rd week the sheep were harnessed for the collection of faeces and urine. The sheep were then fasted for 10 d and allowed access to water throughout this period. Daily samples of urine were taken and the output of faecal dry matter

Table 1. *Expt* 1. *Dry-matter and nitrogen intakes of sheep before starvation*

Sheep no.	Ration	Dry matter (g/24 h)	N (g/24 h)
1	Hay	840	7·0
2	Dried grass	1191	24·3
3	Hay	840	7·0
4	Dried grass	929	18·9

Table 2. *Expt* 2. *Dry-matter and nitrogen intakes of sheep and the amounts of casein infused through ruminal or abomasal fistulas*

Ration	Sheep no.	Dry-matter intake (g/24 h)	N intake (g/24 h)	Casein N infused* (g/24 h)							
				Rumen				Abomasum			
				P 1	P 2	P 3	P 4	P 1	P 2	P 3	P 4
Hay	5	676	9·0	7·2	13·6	—	—	7·7	13·5	—	—
Hay	9	676	9·0	—	13·7	—	—	—	—	—	—
Hay	7	678	8·7	6·9	13·7	20·0	25·3	—	—	—	—
Hay–maize†	7	512	8·2	7·3	13·6	—	—	7·3	14·7	—	—
Hay–maize†	4	512	8·2	—	—	19·7	26·5	—	—	—	—
Hay–maize†	3	495	8·0	7·5	14·5	19·4	24·8	—	—	—	—
Dried grass	12	755	16·5	—	—	—	—	7·0	13·0	19·9	23·0
Dried grass	13	755	16·5	—	—	—	—	6·2	12·7	18·5	22·5
Dried grass	14	755	16·5	—	—	—	—	6·2	12·0	18·0	22·5

* In periods P 1, P 2, P 3 and P 4, 2 l of 25, 50, 75 or 100 g/l solutions of casein in saline (see below) respectively were infused by continuous drip in 24 h.

† Contained 200 g hay and 800 g maize/kg on a fresh-weight basis.

was determined. Composite 7 d samples of faeces and urine were prepared as described previously (Martin, 1970) during the week before starvation.

Experiment 2

The eight sheep used were maintained on one of the three rations described in Table 2 for a period of 3 weeks before being harnessed for faeces and urine collection. All the experimental periods were of 7 d duration. In initial control periods 2 l/24 h of the saline solution described by Blaxter & Martin (1962) were infused as a continuous drip into the rumen or abomasum. Control periods were repeated until a stable urinary output of aromatic acids was obtained. After the end of the control periods, 2 l/24 h of 25, 50, 75 or 100 g/l solutions of casein (Light White Soluble; British Drug Houses Ltd, Poole, Dorset) in saline solution were infused as a continuous drip into the sheep by the same route as was the saline solution in the preceding control periods. After two or four casein-infusion periods in which the concentration of casein infused was increased in succeeding periods, two further control periods followed in which the saline solution was infused. In casein-infusion periods and control periods immediately following them composite samples of faeces and urine were prepared from the collections on the final 4 d of each period only. In all other control periods composite samples of faeces and urine were prepared from samples

Table 3. *Expt* 3. *Dry-matter, nitrogen and phenylalanine intakes* (*g*/24 *h*) *by sheep on the hay–sugar-beet pulp* (*HS*), *hay–rolled barley* (*HR*), *hay–linseed meal* (*HL*) *and hay–field beans* (*HB*) *rations**

	HS	HR	HL	HB
Dry matter	1278	1100	709	803
N	18·6	15·6	22·1	23·0
Phenylalanine	2·93	4·18	5·45	5·52

* Hay supplied 428 g dry matter, 4·4 g N and 1·15 g phenylalanine in all rations.

Table 4. *Expt* 3. *Sequence of experimental rations**

Period no.	Sheep no. 6	Sheep no. 8	Sheep no. 10	Sheep no. 11
1	HS	HR	HL	HB
2	HL	HB	HS	HR
3	HB	HS	HR	HL
4	HR	HL	HB	HS

* HS, hay–sugar-beet pulp; HR, hay–rolled barley; HL, hay–linseed meal; HB, hay–field beans.

collected on all 7 d. Casein was infused into the rumen of sheep during fifteen experimental periods and into the abomasum of sheep during sixteen experimental periods. The composition of the diets consumed by the sheep and the amounts and route by which casein nitrogen was infused in all of the thirty-one casein infusion periods are presented in Table 2. Food was offered to all sheep in two equal portions at 06.30 hours and 16.30 hours daily.

Experiment 3

Four rations were prepared for this experiment by adding to a constant weight of hay either sugar-beet pulp (HS), rolled barley (HR), extracted linseed meal (HL) or field beans (HB). It was intended that the rations should contain equal amounts of N but the sheep would not eat the amounts of sugar-beet pulp and rolled barley required to achieve this aim. Therefore the amounts of sugar-beet pulp and rolled barley in diets HS and HR respectively were reduced. Four sheep were used (nos 6, 8, 10 and 11) and each received each of the four rations in turn for a period of 4 weeks. At the end of the 3rd week of the feeding period they were harnessed for faeces and urine collection and composite samples of faeces and urine were prepared from those collected during the final 5 d of each period. The composition and intake of dry matter and N by the sheep given these rations is given in Table 3. The sequence in which the rations were offered to the sheep is recorded in Table 4. Food was offered in two equal portions daily as in Expt 2.

Sheep no. 11 had a rumen fistula and during the 3rd week of feeding samples of rumen liquor were withdrawn immediately before feeding and at 45 min, 1·5 h, 2·5 h, 3·5 h, 5·0 h and 7·0 h after feeding for the analyses described below.

Foods

Methods of analysis

Nitrogen. Kjeldahl digestion of the sample (1 g) using copper (412 mg $CuSO_4 . 5H_2O$) and selenium (40 mg) catalysts was followed by distillation of a portion (1 %) of the digest in a Markham still. Ammonia was absorbed in boric acid and titrated with standard acid.

Phenylalanine. Samples of food were hydrolysed with 6 M-hydrochloric acid and phenylalanine was determined by means of an automatic amino acid analyser (Evans Electroselenium Ltd, Halstead, Essex).

Urine

Hydrolysis of conjugated acids. Urine (0·5–2·0% of 24 h output) was hydrolysed with KOH as described previously (Martin, 1970).

Diethyl ether-soluble acids of hydrolysed and unhydrolysed urine. These acids were determined as described previously (Martin, 1969*a*). The results were expressed in terms of acidity equivalent to benzoic acid (BAE).

Extraction of aromatic acids. In Expts 1 and 2 these acids were determined by continuous extraction with light petroleum (b.p. 40°–60°) as previously described (Martin, 1969*a*). However, toluene was found to be a more efficient solvent than light petroleum (b.p. 40°–60°) both for benzoic acid (partition coefficients between toluene and water and between light petroleum and water were 0·80 and 0·62 respectively) and phenylacetic acid (partition coefficients between toluene and water and between light petroleum and water were 0·67 and 0·22 respectively).

In Expt 3 the following method was adopted. The sample (which contained up to 100 mg BAE) was pipetted into a separating funnel, acidified to pH 2 with 6 M-HCl and NaCl was added (2 g/10 ml). This solution was extracted three times with half its volume of toluene and the toluene phases were combined and dried over anhydrous sodium sulphate. The toluene extract was made to a known volume and half of the extract evaporated to dryness in a rotary film evaporator. The residue was taken up in warm water and the total aromatic acids were determined by titration of the solution to pH 7·6 with 0·1 M-NaOH using an automatic titrator (Radiometer, Copenhagen, Denmark). The remaining toluene extract was used for gas–liquid chromatographic assay of the aromatic acids.

Glycine conjugates of aromatic acids. In Expt 1 hippuric acid was determined by the thin-layer chromatographic method described by Martin (1969*b*). Some difficulty was experienced when this method was used to determine the hippuric acid content of urines from sheep which had been fasted for 4 or more d. The hippuric acid content of these urines was up to 200 times less than that of urines from fed sheep. Extracts equivalent to proportionally greater amounts of the total daily urine output had therefore to be applied to thin-layer plates when the hippuric acid content of fasted urines was determined. On spraying thin-layer plates with *p*-dimethylaminobenzaldehyde in acetic anhydride (to form hippuric acid azlactone) a number of unidentified spots, some of which were not resolved from hippuric acid, appeared on those plates to which it had been necessary to apply comparatively large amounts of urine

Table 5. *Molecular extinctions* ($\epsilon \times 10^{-3}$) *of benzoic, phenylacetic, 3-phenylpropionic and cinnamic acids in* 0·01 M-*NaOH and* 0·01 M-*HCl at* 225 *and* 230 *nm*

	0·01 M-NaOH	0·01 M-HCl
Wave length of benzoic acid maximum absorption (nm)	225	230
Benzoic acid	8·19	10·79
Phenylacetic acid	1·33	0·34
3-Phenylpropionic acid	0·06	0·03
Cinnamic acid	6·33	2·10

extract. This caused some overestimation of the hippuric acid content of the urine of fasted sheep.

A gas–liquid chromatographic assay of these compounds was therefore developed. For this purpose phenaceturic acid was synthesized from glycine and methylphenylacetate by the method described by Ford (1949). Methanolic solutions of the pure acids or of the residues of diethyl ether extracts of unhydrolysed urine were mixed with a solution of heneicosanoic acid in chloroform (5 mg/ml), which was used as an internal standard. Portions of this solution containing 0·2 mg of internal standard and 0·04–1·0 mg of glycine conjugates of aromatic acids (as BAE) were methylated with 1 ml of a solution of diazomethane in diethyl ether (prepared from diazald; R. N. Emanuel & Co., Wembley, London). Excess diazomethane and diethyl ether were removed from the tube containing the methylated acids by passing a stream of dry N_2 over the surface of the liquid. A model 64 Pye 104 dual-flame ionization gas chromatograph (Pye Unicam Ltd, Cambridge) fitted with glass columns (1520 mm long, 4 mm i.d.) which were packed with a neopentylglycol succinate-coated support (50 g/kg on 80–100 mesh Universal B; Phase Separations Ltd, Flintshire), was used. Chromatographic conditions were as follows: oven temperature, isothermal 195°; injection port temperature, 210°; detector oven temperature, 350°; carrier gas, argon at an inlet pressure of 3·2 bar and flow rate of 45 ml/m; amplifier attenuation was varied between 10×10^2 and 50×10^2. Samples containing 2·5 μg of internal standard were injected on to the column. Relative retention volumes of the methyl esters of benzoic, phenylacetic, cinnamic, hippuric and phenaceturic acids to the internal standard were 0·02, 0·03, 0·10, 1·32 and 1·61 respectively: the approximate retention time of the internal standard was 35 min. Known mixtures of the glycine conjugates and internal standard were prepared and graphs plotted of the peak area ratio of the acids to the internal standard against the corresponding weight ratios of the glycine conjugates to the internal standard. Areas were determined by triangulation. The graphs were used to calculate the amounts of acids in urine extracts; the graphs were linear between weight ratios of 0·6 and 4·0.

Aromatic acids in hydrolysed urine. The u.v. spectrophotometric assay of benzoic acid described by Martin (1969*b*) was modified and used in Expts 1 and 2. The alkaline extracts containing the aromatic acids were acidified before scanning the u.v. absorption spectra between 300 and 210 nm. This induced a bathochromic shift in the absorption maximum of benzoic acid of 5 nm and also had a hyperchromic effect, as reported in Table 5. Interference from other aromatic acids was also reduced.

The distribution of the total aromatic acids determined by titration in Expts 1 and 2 was determined by gas–liquid chromatography of the free acids on columns of Carbowax 20 M/TPA (Martin, 1970). In fifty-six analyses the benzoic acid content of samples analysed by u.v. spectrophotometry and by titration of light petroleum extracts followed by gas–liquid chromatography was compared: benzoic acid determined by the former method was $98{\cdot}7 \pm 1{\cdot}7\%$ of that determined by the latter method (mean and standard error).

In Expt 3 aromatic acids were quantitatively assayed by gas–liquid chromatography alone using the chromatograph and columns described for hippuric and phenaceturic acids. Portions of the toluene extract of aromatic acids containing 25 mg BAE were evaporated to dryness in a rotary film evaporator and the residue was taken up in 2 ml of a toluene solution of 10-undecenoic acid (10 mg/ml) which was used as an internal standard. This solution (1 ml) was methylated with a solution of diazomethane in diethyl ether (1 ml) and 1 μl was injected on to the column. Chromatographic conditions which differed from those described for glycine conjugates were: oven temperature, 115°; injection port temperature, 135°. Relative retention volumes of the methyl esters of benzoic, phenylacetic, 3-phenylpropionic and cinnamic acids to the internal standard were 0·38, 0·76, 1·21 and 3·10 respectively. Response factors relative to the internal standard were determined as described for the glycine conjugates of aromatic acids.

Combined glucuronides. The method described by Fishman & Green (1955) was used in Expt 1.

Column chromatography of aromatic acids and creatinine. Sinha & Gabrieli (1968) used columns (400 mm long, 10 mm diameter) containing G-10 Sephadex (Pharmacia, Uppsala, Sweden) to separate creatinine, hippuric acid and benzoic acid. Samples containing 0·4 mg creatinine and various acids in 0·1 M-phosphate buffer of pH 7 were applied to those columns which were eluted with the same buffer at a rate of 0·4 ml/m (Varioperpex pump; LKB, Sweden). The absorbance of the eluate was monitored at 233 nm using a 0·2 mm path-length flow-cell in an SP 800 spectrophotometer (Pye Unicam Ltd, Cambridge). Phenylacetic acid, phenaceturic and hippuric acids all eluted in the same peak (maximum 34 ml; range 30–42 ml). Though the method did not give good separations of aromatic acids it was used for creatinine assay. The only acids found to co-elute with creatinine were acetic, propionic and *n*-butyric acids which however do not absorb light at 233 nm, which is the wavelength of maximum absorbance for creatinine in pH 7 phosphate buffer. In Expt 3 creatinine was determined by measuring the area of the creatinine peak by triangulation and reference to a standard curve produced when known weights of creatinine were applied to the columns.

Nitrogen. Samples (2 ml) were analysed as described for food N but 5% of the Kjeldahl digest was used for distillation.

Faeces

Nitrogen. The method described for urine was used.

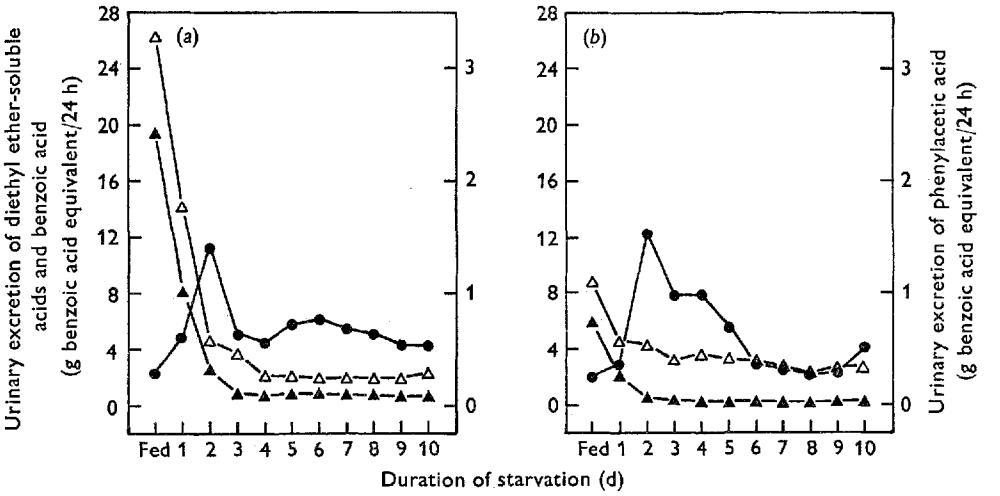

Fig. 1. Diethyl ether-soluble acids (△), benzoic acid (▲) and phenylacetic acid (●) extracted from the hydrolysed urine of (*a*) sheep no. 2 and (*b*) sheep no. 3 which were fed on dried grass and hay respectively and then starved for 10 d.

Rumen liquor

Ammonia. Rumen liquor was strained through muslin and 5 ml were mixed with 5 ml 0·1 M-HCl in a 25 ml volumetric flask and made to the mark with water. The ammonia content of this solution was determined by the microdiffusion method described by Conway (1947).

Aromatic acids. Strained rumen liquor was centrifuged for 15 min at 12000 *g*. The clear supernatant fraction was removed, and 50 ml were acidified to pH 1 and extracted with toluene as described above. The combined toluene phases were washed three times with half their volume of sodium bicarbonate (50 g/l) and the acids were transferred to diethyl ether by acidification of the bicarbonate and extraction three times with an equal volume of diethyl ether. The combined ether solutions were dried over sodium sulphate and then taken to dryness in a rotary film evaporator. The aromatic acids were determined by gas–liquid chromatography of their methyl esters as described above.

RESULTS

Experiment 1

Weight of animals. On the last day of feeding and on the 5th and 10th days of fasting the weights of the sheep were respectively: sheep no. 1, 52·0, 45·8 and 42·4 kg; sheep no. 2, 63·4, 60·6 and 56·5 kg; sheep no. 3, 72·9, 65·2 and 58·7 kg; sheep no. 4, 64·0, 57·7 and 54·6 kg.

Starvation and urinary acid excretion. On withholding food the urinary excretion of all acids except phenylacetic acid declined rapidly; the rate of decline was greater for the sheep given dried grass (nos 2 and 4) than for those given hay (nos 1 and 3). The daily urinary output of diethyl ether-soluble, benzoic and phenylacetic acids found on analysis of hydrolysed urines of sheep no. 2 and sheep no. 3 are shown in

Table 6. *Mean daily urinary excretion of acids by sheep between the 5th and 10th days of starvation*

	Sheep no. 1	Sheep no. 2	Sheep no. 3	Sheep no. 4	Standard error (residual mean square)	Difference between means required for $P < 0.05$
Diethyl ether-soluble acids of hydrolysed urine*	1·80	2·00	2·80	1·95	0·11	0·45
Diethyl ether-soluble acids of unhydrolysed urine*	1·41	1·41	1·94	1·39	0·08	0·34
Phenolic+ aliphatic acids*†	1·31	1·27	2·05	1·43	0·11	0·42
Total benzoic acid*	0·15	0·08	0·18	0·09	0·01	0·04
Hippuric acid*	0·13	0·15	§	0·19	0·02	0·05
Total phenylacetic acid*	0·38	0·65	0·41	0·43	0·05	0·21
Combined glucuronides‡	§	0·67	1·19	0·78	0·05	0·20

* g benzoic acid equivalent/24 h.
† Difference between diethyl ether-soluble and light petroleum (b.p. 40°–60°)-soluble acids of hydrolysed urine (see below).
‡ g glucuronic acid/24 h.
§ Not determined.

Fig. 1. Analysis of variance of the daily urinary output of urinary acids by fasted sheep showed that after the 4th day of starvation there was no change in excretion of any of the acids analysed. However, sheep no. 3 excreted significantly greater quantities ($0.01 < P < 0.05$) of diethyl ether-soluble acids of hydrolysed and unhydrolysed urine, of benzoic acid, of phenolic + aliphatic acids and of combined glucuronides than did sheep nos 1, 2 and 4. Adjustment of these results on the basis of metabolic body size (kg $W^{0.73}$) did not remove these differences. The mean daily excretion by each sheep from the 5th to the 10th day of starvation of the various urinary acids determined are presented in Table 6.

Diethyl ether-soluble acids. When sheep were fed, hydrolysis of the urine increased the acids extracted by diethyl ether by factors of 1·06 for sheep no. 4 and 1·11 for sheep nos 1, 2, and 3. During the final 6 d of starvation hydrolysis of urine increased the acids extracted by diethyl ether by a factor of 1.40 ± 0.08 (mean and standard error).

Phenolic plus aliphatic acids. In addition to aromatic acids (determined by light petroleum or toluene extraction), diethyl ether extracts almost all phenolic acids (Williams & Sweeley, 1964) and some aliphatic acids, e.g. lactic and succinic acids are extracted completely on 16 h continuous extraction, but other acids such as citric and oxalic acids are only partly extracted (Martin, unpublished observations). The difference between the total diethyl ether-soluble acids of hydrolysed urine is due to the phenolic and some of the aliphatic acids excreted in urine.

When the animals were fed, the proportions of the diethyl ether-soluble acids of hydrolysed urine that were excreted by sheep nos 1–4 as phenolic + aliphatic acids respectively were 0·30, 0·29, 0·28 and 0·51. During the final 6 d of fasting, analysis of variance showed no difference due either to sheep or duration of fast in the proportion

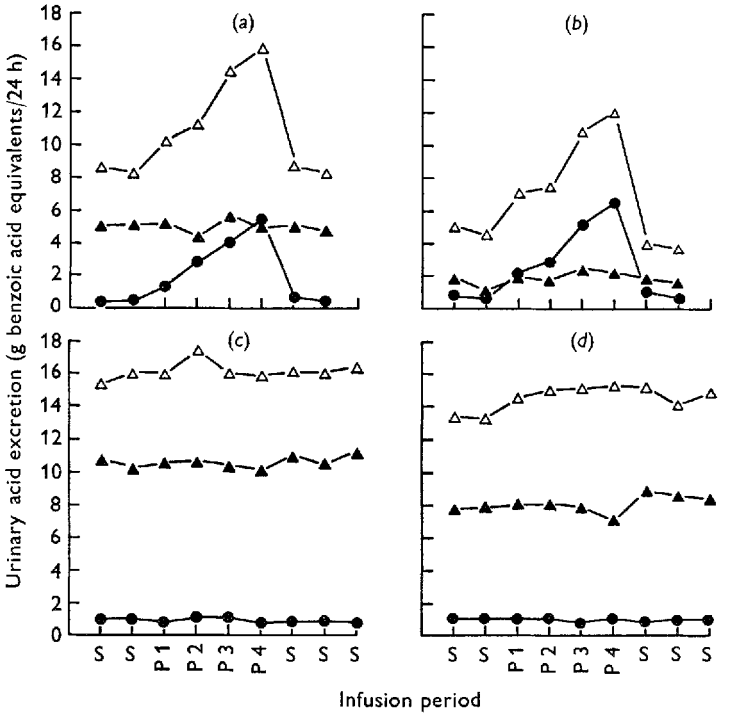

Fig. 2. Diethyl ether-soluble acids (△), benzoic acid (▲) and phenylacetic acid (●) extracted from the hydrolysed urines of (*a*) sheep no. 7 and (*b*) sheep no. 3 when they received ruminal infusions of saline or casein and of (*c*) sheep no. 12 and (*d*) sheep no. 14 when they received abomasal infusions of saline or casein. Infusions: S, 2 l saline/24 h; P 1, P 2, P 3 and P 4, 2 l/24 h respectively of 25, 50, 75 and 100 g/l solutions of casein in saline. Rations: sheep no. 7, hay; sheep no. 3, flaked maize–hay, 4:1 (w/w); sheep nos 12 and 14, dried grass.

of diethyl ether-soluble acids of hydrolysed urine represented by acids of this fraction, namely 0·71 ± 0·02 (mean and standard error).

Benzoic acid, hippuric acid and combined glucuronides. The wide variation found in urinary benzoic acid excretion by sheep when the sheep were fed (5·37 g BAE/d by sheep no. 1 and 19·42 g BAE/d by sheep no. 3) did not affect the time required for achievement of a stable level of urinary excretion when fasted (Fig. 1). Hippuric acid excretion by two sheep (nos 2 and 4) was apparently greater (in terms of BAE) than that of benzoic acid when the sheep were fasted; reasons for this have already been noted (p. 255).

Urinary excretion of combined glucuronides followed a pattern similar to that found for benzoic and hippuric acids. Sheep nos 2 and 4 excreted 5·5 and 6·4 g of combined glucuronides/d when given dried grass and sheep no. 3, given hay, excreted 3·8 g/d. Daily urinary output declined during the first 4 d of fasting and did not change significantly thereafter.

Phenylacetic acid. The mean urinary output of this acid when sheep nos 1–4 were given food was 0·25 g BAE/d. When fasted, the amounts of phenylacetic acid extracted from hydrolysed urine samples of all four sheep increased to a maximum (mean 1·24 g BAE/d) between the 1st and 4th days of fasting. Although further

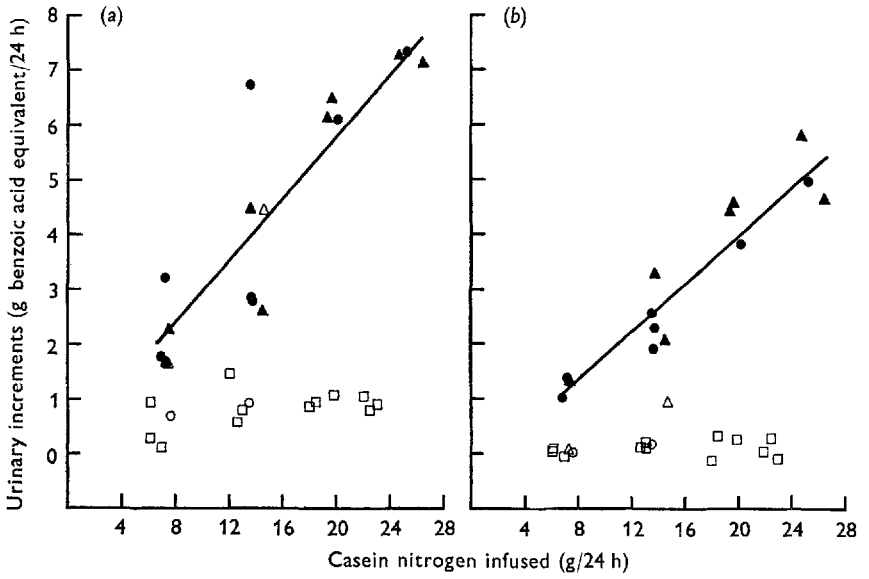

Fig. 3. Increments found in hydrolysed urine of (*a*) diethyl ether-soluble acids and (*b*) phenylacetic acid when solutions of casein were infused into the rumen (solid symbols) or abomasum (open symbols) of sheep. Rations given to sheep: circles, hay; triangles, 4:1 (w/w) flaked maize–hay; squares, dried grass. The regression equations of diethyl ether-soluble acid increments (E) and phenylacetic acid (PA) on casein N infused (P) are shown and were: $E = 0{\cdot}28P + 0{\cdot}16$; $PA = 0{\cdot}22P - 0{\cdot}41$; Both were significant at $P < 0{\cdot}001$.

starvation reduced the amounts of phenylacetic acid excreted, the amount throughout the 10 d fasting period was as great or greater than that observed when the sheep were fed (see Fig. 1).

Experiment 2

In Fig. 2 the quantities of diethyl ether-soluble, benzoic and phenylacetic acids found in hydrolysed urine in two experiments in which casein was given by ruminal infusion (sheep nos 7 and 3) and two experiments in which casein was given by abomasal infusion (sheep nos 12 and 14) are presented. Ruminal infusion of casein caused large increases in urinary excretion of diethyl ether-soluble acids, the greater part of which was due to increments in urinary phenylacetic acid. Benzoic acid excretion showed no significant change on ruminal infusion of casein; in fifteen experiments a mean increment of 19 ± 14 mg BAE/g N infused (mean and standard deviation) was observed.

With one exception, abomasal infusion of casein did not cause any significant change in urinary excretion of any of the acids examined (Fig. 2). Only in one of the sixteen abomasal infusion experiments was any change in urinary acid excretion observed, and during this period the animal (sheep no. 7) showed evidence of considerable digestive disorder by scouring badly. Increments of diethyl ether-soluble acids and phenylacetic acid observed in hydrolysed urine were 4·45 and 0·91 g BAE/d respectively.

The relationships between increments in the diethyl ether-soluble and phenylacetic

Table 7. *Mean intake of apparently digestible nitrogen (g N/24 h) and mean urinary excretion (g BAE/24 h) of diethyl ether-soluble acids, benzoic acid and phenylacetic acid by sheep given four different dietary sources of protein*

Ration*	Apparently digestible N intake	Diethyl ether-soluble acids: Hydrolysed urine	Diethyl ether-soluble acids: Unhydrolysed urine	Phenolic and aliphatic acids	Benzoic acid	Phenyl-acetic acid
HS	11·14	10·76	8·70	5·35	4·93	0·26
HR	9·05	7·45	5·87	3·81	2·92	0·49
HL	16·63	10·73	9·69	3·07	5·54	2·04
HB	17·45	8·09	7·25	3·14	2·88	1·99
Standard error of treatment means	0·27	0·43	0·41	0·35	0·12	0·08
Difference between means required for significance $P<0·01$	1·40	2·26	2·14	1·84	0·65	0·44

BAE, benzoic acid equivalent.
* See Tables 3 and 4 for description of rations.

acid contents of hydrolysed urine and the amounts of casein infused into the rumen are illustrated in Fig. 3, which also shows the corresponding values when casein was infused into the abomasum. Significant linear regressions ($P < 0·001$) were found for both diethyl ether-soluble acids and phenylacetic acid on ruminal infusion of casein; the percentage of the total variance accounted for by the two regressions was 76 for diethyl ether-soluble acids and 86 for phenylacetic acid.

Experiment 3

Weights of sheep. All sheep were weighed at the beginning and end of the 7 d period in which faeces and urine were collected. The means of these weights (kg) of the sheep during periods when HS, HR, HL and HB were given were respectively: sheep no. 6, 74·2, 70·6, 69·1 and 68·0; sheep no. 8, 78·4, 77·8, 74·6 and 75·7; sheep no. 10, 73·8, 75·0, 68·6 and 73·3; sheep no. 11, 66·5, 63·9, 62·3 and 62·7. All sheep gained weight during periods HS and HR and lost weight during periods HL and HB.

Creatinine excretion. Daily assays of urinary creatinine were used as an index of the completeness of 24 h collections of urine. When values persistently lower than the average output were obtained the reason for the loss was ascertained and the collection period extended until five consecutive samples showed a satisfactory average creatinine content. The experimental design was a Latin square (Table 4), and statistical analysis showed no significant difference in urinary creatinine excretion due either to ration or to the sequence in which the rations were offered. The average creatinine excretion of the four sheep was $1·52 \pm 0·08$ g/d.

Urine acids. Statistical analysis showed no significant differences due either to sheep or to the sequence in which the rations were offered for any of the urine acids examined. There were significant differences due to the ration: significance levels were

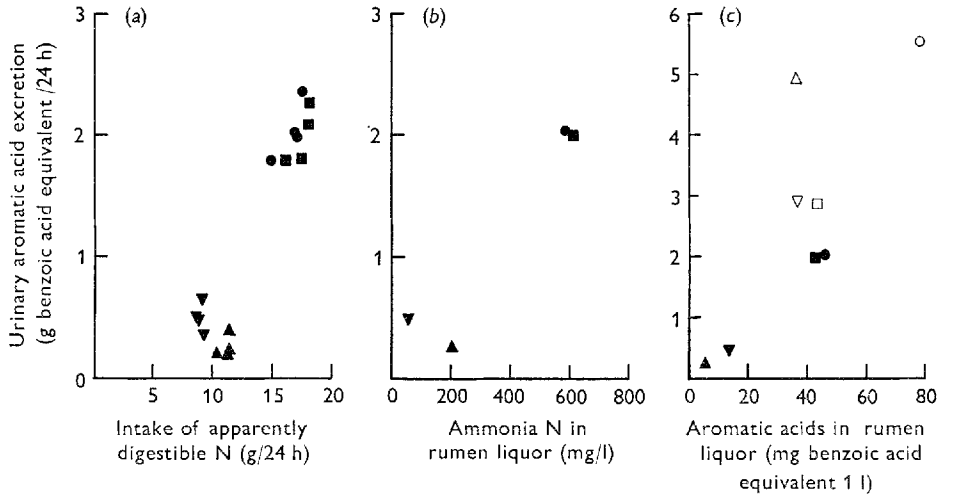

Fig. 4. Relationships between the urinary aromatic acid excretions of sheep nos 6, 8, 10 and 11 and (*a*) their intakes of apparently digestible nitrogen, (*b*) the mean concentration of ammonia N in the rumen liquor of sheep no. 11 and (*c*) the mean concentration of aromatic acids in the rumen liquor of sheep no. 11. Rations: hay–sugar-beet pulp, △; hay–rolled barley, ▽; hay–linseed meal, ○; hay–field beans, □. Open symbols, urinary benzoic acid and ruminal 3-phenylpropionic acid; closed symbols, urinary and ruminal phenylacetic acid.

$0{\cdot}001 < P < 0{\cdot}01$ for diethyl ether-soluble acids of both hydrolysed and unhydrolysed urine, $0{\cdot}01 < P < 0{\cdot}05$ for phenolic + aliphatic acids and $P < 0{\cdot}001$ for both benzoic and phenylacetic acids. Mean results for these fractions are presented in Table 7. The effects of diet on urinary excretion of both diethyl ether-soluble acids and benzoic acid were similar; rations HS and HL resulted in significantly greater excretion ($0{\cdot}001 < P < 0{\cdot}01$) than rations HB and HR. Ration HS caused a significantly greater excretion of acids of the phenolic + aliphatic acid fraction ($0{\cdot}001 < P < 0{\cdot}01$) than all other rations. In contrast, ration HS led to the smallest observed urinary excretion of phenylacetic acid. Sheep given rations HL and HB excreted significantly greater amounts of phenylacetic acid ($P < 0{\cdot}001$) than did sheep consuming rations HS and HR.

Apparent digestibility of protein and urinary excretion of phenylacetic acid. There were no significant differences in the apparent digestibility of N due either to sheep or to the sequence of feeding with the four rations but there were significant differences between rations. The mean apparent digestibility ratios of N in rations HS, HR, HL and HB were 0·60, 0·58, 0·75 and 0·76 respectively; rations HS and HR had significantly lower digestibility ratios ($P < 0{\cdot}001$) than rations HL and HB. In Fig. 4(*a*) the urinary phenylacetic acid excreted by all sheep is plotted against their intake of apparently digestible N.

Ammonia and aromatic acids in rumen liquor. One rumen-fistulated sheep was available for these experiments (no. 11) and the ammonia-N and aromatic acid contents of the rumen liquor sampled at the previously noted times (p. 254) were determined.

When sheep were given diet HR the concentration of rumen ammonia was small and varied only slightly with time of sampling. When given diet HS, HL or HB the

concentrations of rumen ammonia increased to maxima that were between 100 and 270 mg ammonia-N/l greater between 1·5 and 3·5 h after feeding than immediately before feeding. In Fig. 4(*b*) the mean NH_3-N found in all samples for each ration is plotted against the mean urinary phenylacetic acid excretion of the sheep used in this experiment.

The principal aromatic acids found in rumen liquor were 3-phenylpropionic acid and phenylacetic acid with small amounts of benzoic (up to 6 mg BAE/l) and cinnamic (up to 7 mg BAE/l) acids. With all four diets the concentration of 3-phenylpropionic acid increased slowly after feeding to a maximum observed between 3·5 and 7 h later. With rations HB and HL phenylacetic acid concentration varied in a similar manner, but when rations HS and HR were given phenylacetic acid concentration exceeded 10 mg BAE/l only in samples obtained immediately before and up to 2·5 h after feeding. In Fig. 4(*c*) the mean (of all samples taken between feeds) rumen liquor concentrations of 3-phenylpropionic acid are plotted against the mean daily output of benzoic acid by all sheep used in this experiment: similar results are plotted for ruminal concentrations of phenylacetic acid and urinary phenylacetic acid excretion.

DISCUSSION

Urinary output of aromatic acids by fasted sheep

After 4 d of fasting the urinary output of diethyl ether-soluble acids of unhydrolysed and of hydrolysed urine, or phenolic + aliphatic acids, of benzoic acid and of phenylacetic acid were similar to those reported previously after this period of fast (Martin, 1969*b*). The present results show that an additional day of fast is necessary before stable outputs of these acids are attained. The failure of 10 d periods of fasting to cause reductions in the comparatively large amounts of urinary phenylacetic acid excreted during the final 6 d of fasting is difficult to explain. Small amounts of phenylacetic acid are produced as an end-product of a minor pathway of phenylalanine catabolism via phenylpyruvate (Moldave & Meister, 1957). However, abomasal infusion of casein containing up to 7·18 g of phenylalanine to sheep did not result in a significant increase in urinary phenylacetic acid excretion (Fig. 3(*b*)); increments of 255 mg of phenylacetic acid would have been significant ($0{\cdot}05 < P < 0{\cdot}01$) which shows that less than 4·3% of the phenylalanine infused was catabolized to phenylacetic acid.

Even on prolonged fasting of ruminant animals, a considerable amount of digesta has been found to remain in the rumen (Hungate, 1966). In the present experiments output of faecal dry matter was maintained at an appreciable level during the first 4 d of fasting (mean 110 g/d), which coincided with the period in which maximum urinary excretion of phenylacetic acid was observed. During the remaining 6 d of fasting the pattern of faeces output was irregular, but an average of 23 g dry matter/d was excreted. Even after 10 d starvation, continuing fermentation of digesta remaining in the rumen is the most probable source of the urinary phenylacetic acid excreted by sheep.

Protein metabolism and urinary aromatic acid excretion

On ruminal infusion of between 6·9 and 26·5 g casein N/d, analysis of hydrolysed urine showed increments in diethyl ether-soluble acids and phenylacetic acid of 284 ± 44 and 220 ± 21 mg BAE/g casein N respectively (regression coefficients and their standard errors; Fig. 3). The phenylalanine content of the casein infused was 312 mg/g N. Scott *et al.* (1964) reported that phenylalanine was the only amino acid to yield phenylacetic acid on microbial degradation in the rumen. The incremental phenylacetic acid found in the urine on ruminal infusion of casein thus accounted for 95% of the phenylalanine contained in the casein. Only in one sheep (no. 7) which was scouring did abomasal infusion of casein cause an increment in the diethyl ether-soluble acids (303 mg BAE/g N) or phenylacetic acid (63 mg BAE/g N) content of hydrolysed urine. This suggests that protein digestion and absorption are completed before fermentable protein has reached the microbial population of the large intestine and caecum and that phenylacetic acid production in the normal sheep is confined to protein degradation in the rumen.

No increment in benzoic acid excretion was observed in any of the present experiments in which casein was infused into sheep. This contrasts with observations of Scott *et al.* (1964), who found that tyrosine was metabolized to 3-(*p*-hydroxyphenyl)-propionic acid by rumen micro-organisms and subsequently dehydroxylated to 3-phenylpropionic acid, which was absorbed and excreted as benzoic acid. Increments of 64 mg BAE/g casein N infused into the rumen were observed in the phenolic + aliphatic acid fraction of urinary acids. These acids were not identified but probably included metabolites of tyrosine and tryptophan. Further studies are in progress on the metabolism of tyrosine and tryptophan by rumen micro-organisms.

Virtually all the benzoic and phenylacetic acids excreted in the urine of sheep was conjugated with glycine. In experiments in which casein was infused into the rumen $99{\cdot}1 \pm 9{\cdot}3\%$ and $101{\cdot}7 \pm 6{\cdot}5\%$ (mean and standard deviations) of the conjugated benzoic and phenylacetic acids excreted were found to be hippuric and phenaceturic acids respectively; only 2·9% and 2·5% respectively of the total benzoic and phenylacetic acids were excreted unconjugated.

The products of proteolysis in the rumen may be utilized for microbial protein synthesis if conditions for rapid microbial growth are favourable. Thus, Annison, Chalmers, Marshall & Synge (1954) have shown that the extent of ammonia production in the rumen from readily fermented proteins may be reduced by supplementing the diet with a readily fermentable carbohydrate. Allison (1965) has shown that rumen bacteria can utilize phenylacetic acid for phenylalanine biosynthesis. In Expt 3 two of the rations (HS and HR) given to sheep contained a large amount of readily fermentable carbohydrate (gross energy:N in ration, 1256 kJ (300 kcal/g N)) and two others (HL and HB) a smaller amount of carbohydrate (gross energy:N in ration, 628 kJ (150 kcal)/g N).

Of the urinary fractions examined (see Table 7) only phenylacetic acid excretion was related to N intake, which confirms the observations of previous experiments (Martin, 1969*a*, 1970). Increased urinary phenylacetic acid excretion was associated

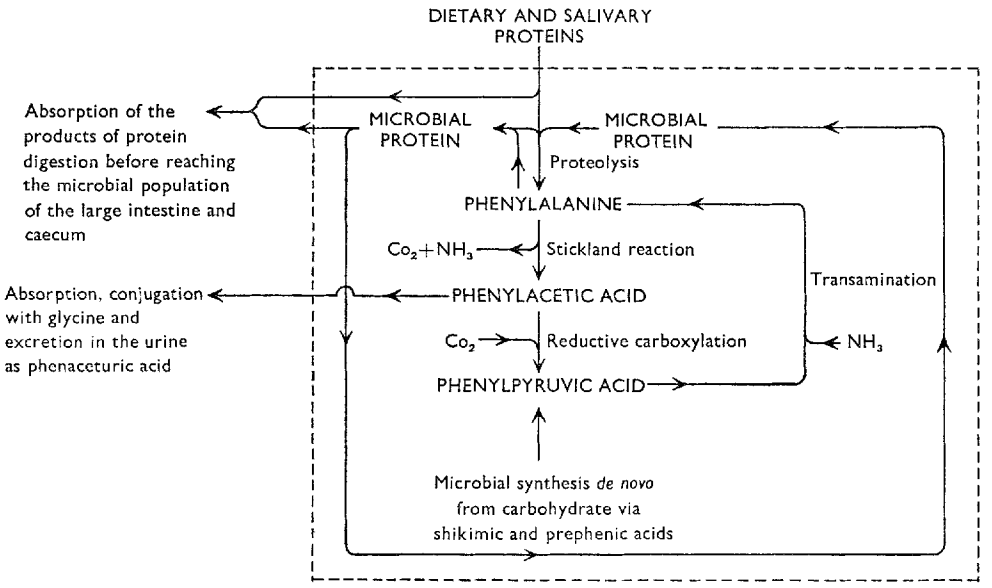

Fig. 5. Diagram illustrating the metabolism of phenylalanine in the rumen. — —, Rumen wall.

with corresponding increases in ruminal ammonia N and ruminal phenylacetic acid concentrations (Fig. 4*b*, *c*) and in apparently digestible N intakes (Fig. 4*a*), the latter increases being the most marked. Indeed, had the apparently digestible N intake of the sheep given the four rations used in this experiment been the same, it is possible that no such relationship would have existed. Although only limited results were available on ruminal metabolism in Expt 3, those presented in Fig. 4 support the hypothesis that the major source of urinary phenylacetic acid is microbial metabolism of protein in the rumen, rather than digestion of protein on subsequent passage through the digestive tract. The percentages of dietary phenylalanine that was excreted as urinary phenylacetic acid was least for the two sheep consuming the high-energy rations HS and HR, namely 11 and 16 respectively; the sheep consuming rations HL and HB excreted 51 and 49 respectively of their phenylalanine intake as phenylacetic acid. Thus, when conditions favoured rapid microbial growth the loss of dietary phenylalanine as phenylacetic acid was small. However, the presence of a supply of readily available energy did not restrict ruminal phenylacetic acid excretion in all experiments; similar increments of urinary phenylacetic acid were observed/g casein infused into the rumen of sheep maintained on either roughage (hay) or concentrate (4:1 (w/w) maize–hay) rations (see Fig. 3). Clearly the extent of degradation of soluble protein in the rumen may be more important than the energy content of the ration in determining the amount of phenylacetic acid produced. Mangan (1972) has recently demonstrated that two soluble proteins (casein and ovalbumin) are degraded at very different rates in the rumen of steers.

These experiments have shown that only small amounts of phenylacetic acid are produced by endogenous catabolism of phenylalanine in the body tissues of sheep and that intestinal production of phenylacetic acid by microbial fermentation of dietary

protein in normal sheep is confined to the rumen. In Fig. 5 the known reactions leading to the production and utilization of phenylacetic acid in the rumen are illustrated. It is suggested that the amount of phenylacetic acid excreted by sheep depends on the equilibrium occurring in the rumen between production of phenylacetic acid by microbial degradation of protein and re-utilization of this phenylacetic acid for microbial phenylalanine synthesis (Allison, 1965). Aromatic compounds with a two-carbon side-chain structure are of very limited distribution in plants (Harborne & Simmonds, 1964), and no reports exist of the production of phenylacetic in the intestine from other precursors. Cheng, Krishnamurty, Jones & Simpson (1971) have reported that rumen bacteria metabolize flavan-3-ol glycosides to phenolic phenylacetic acids derived from the B ring of the flavonoid; however, there is no evidence that the phenolic phenylacetic acids are further metabolized to yield phenylacetic acid. Only one flavan-3-ol without any B ring substituents has been reported in plants (Harborne, 1967), and this is rare. Experiments are in progress to determine whether other phenolic precursors yield phenylacetic acid on metabolism in the rumen.

The author is indebted to his colleagues in the Institute for helpful discussions, and in particular to Dr J. H. Moore for his advice and encouragement. Dr W. Manson performed the phenylalanine analyses and Mrs J. T. Knox and Miss E. Colquohoun all the other analyses. The experimental animals were in the care of Mr J. R. McDill and Mr C. Park.

REFERENCES

Allison, M. J. (1965). *Biochem. biophys. Res. Commun.* **18**, 30.
Annison, E. F., Chalmers, M. I., Marshall, S. B. & Synge, R. L. M. (1954). *J. agric. Sci., Camb.* **44**, 370.
Blaxter, K. L. & Martin, A. K. (1962). *Br. J. Nutr.* **16**, 397.
Cheng, K. J., Krishnamurty, H. G., Jones, G. A. & Simpson, F. J. (1971). *Can. J. Microbiol.* **17**, 129.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error* 2nd ed., p. 77. London: Crosby Lockwood & Sons Ltd.
Fishman, W. H. & Green, S. (1955). *J. biol. Chem.* **215**, 527.
Ford, J. H. (1949). *J. Am. chem. Soc.* **71**, 3842.
Harborne, J. B. (1967). *Comparative Biochemistry of the Flavonoids* p. 61. London and New York: Academic Press.
Harborne, J. B. & Simmonds, N. W. (1964). In *Biochemistry of Phenolic Compounds* p. 83 [J. B. Harborne, editor]. London and New York: Academic Press.
Hungate, R. E. (1966). *The Rumen and its Microbes* p. 409. London and New York: Academic Press.
Mangan, J. L. (1972). *Br. J. Nutr.* **27**, 261.
Martin, A. K. (1969*a*). *Br. J. Nutr.* **23**, 389.
Martin, A. K. (1969*b*). *Br. J. Nutr.* **23**, 715.
Martin, A. K. (1970). *Br. J. Nutr.* **24**, 943.
Moldave, K. & Meister, A. (1957). *Biochim. biophys. Acta* **24**, 654.
Patton, S. & Kessler, E. M. (1967). *J. Dairy Sci.* **50**, 1505.
Scott, T. W., Ward, P. F. V. & Dawson, R. M. C. (1964). *Biochem. J.* **90**, 12.
Seakins, J. W. T. (1971). *Clinica chim. Acta* **35**, 121.
Sinha, S. H. & Gabrieli, E. R. (1968). *Clinica chim. Acta* **19**, 313.
Van der Heiden, C., Wadman, S. K., Ketting, D. & De Bree, P. K. (1971). *Clinica chim. Acta* **31**, 133.
Van der Heiden, C., Wauters, E. A. K., Ketting, D., Duran, M. & Wadman, S. K. (1971). *Clinica chim. Acta* **34**, 289.
Wainman, F. W. & Paterson, D. (1963). *J. agric. Sci., Camb.* **61**, 253.
Williams, C. M. & Sweeley, C. C. (1964). In *Biochemical Aspects of Gas Chromatography* p. 238 [H. A. Szymanski, editor]. New York: Plenum Press.

Printed in Great Britain