Fig. 4. Comparison of C_{18} fatty acids in the artificial rumen with and without feeding. The artificial rumen containing 55 ml. of strained rumen contents was given 1 g. of the diet at F: ▲, unsaturated C_{18} acids; ●, stearic acid. A control experiment (no feeding) was also carried out: △, unsaturated C_{18} acids; ○, stearic acid.

similar course of events, with the slow hydrogenation of the unsaturated C_{18} acids occurring at an almost identical rate.

SUMMARY

1. An easily constructed artificial rumen has been designed which is small enough for experi-

ments with valuable isotopically labelled compounds.

2. The rumen micro-organisms are dialysed against flowing clear rumen liquor from the same animal to supply essential growth factors and prevent the excessive accumulation of digestive products.

3. The validity of the apparatus has been proven by using three biochemical criteria, namely the production of volatile fatty acids from cellulose, the release of soluble nitrogen from protein and the hydrogenation of unsaturated fatty acids.

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The Formation and Metabolism of Phenyl-Substituted Fatty Acids in the Ruminant

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The starting point of this investigation was the observation that rumen liquor obtained from sheep contained a fatty acid which partitioned into ether from water and which could not be identified as one of the usual long-chain fatty acids by gas-liquid chromatography. Subsequently this was identified as 3-phenylpropionic acid, and in addition smaller

amounts of phenylacetic acid and benzoic acid were found in the rumen fluid.

3-Phenylpropionic acid was isolated from the rumen contents of the ox by Tappeiner (1886), and since it could also be obtained from decaying protein it was assumed that it was formed by the microbiological decomposition of phenylalanine (Sal-

kowski & Salkowski, 1879; Nawiasky, 1908). Since this time little work appears to have been done on the bacterial formation of the acid.

Phenylacetic acid combined with glycine or glutamine is excreted in the urine of many mammals, and is thought to arise through the transamination of phenylalanine to phenylpyruvic acid which then undergoes oxidative decarboxylation (Henderson, Gholson & Dalglish, 1962); a similar mechanism has been reported in micro-organisms grown under aerobic conditions (Dagley, Fewster & Happold, 1953; Kunita, 1956), but there appears to be no evidence that phenylacetic acid is formed from phenylalanine in anaerobes.

In the present paper a study has been made of the formation of phenyl-substituted fatty acids from aromatic amino acids by the rumen micro-organisms. Both the intact sheep and also the artificial rumen described by Dawson, Ward & Scott (1964) have been used in these experiments, and some attention has also been paid to the subsequent fate of these acids after their absorption from the alimentary tract. A preliminary report of a part of this work has appeared (Scott, Ward & Dawson, 1963).

EXPERIMENTAL

Artificial rumen. This apparatus is described by Dawson *et al.* (1964).

Animals etc. Clun Forest sheep (ewes and wethers) with rumen fistulae were given a constant daily ration of 1000 g. of hay chaff plus 200 g. of oats. Samples of rumen contents were obtained as described by Dawson *et al.* (1964). Blood was taken directly into a syringe containing heparin or through a cannula inserted into the jugular vein. Urine was collected only from wethers with the aid of a large rubber funnel suspended under the animal and draining into a glass reservoir containing 10 ml. of *N*-NaOH to prevent bacterial decomposition of urinary metabolites. Saliva (mainly parotid) was collected by allowing the sheep to chew a piece of pressure tubing connected to a reservoir and suction pump.

Extraction of fatty acids from samples. (a) Rumen contents. The rumen fluid (10–250 ml.) from the intact sheep or the artificial rumen was strained through muslin if necessary and centrifuged for 30 min. at 12 500 *g*_{av.}. The supernatant was adjusted to pH 1 by adding 5*N*-HCl, and extracted twice with equal volumes of ether. In some radioactivity experiments a marker mixture was added at this point containing 1 mg. each of 3-phenylpropionic acid, phenylacetic acid and benzoic acid. The ether phase was washed three times with equal volumes of water to remove any short-chain aliphatic acids that had been extracted from the rumen liquor. The ether solution was then shaken three times with 0.5 vol. of 4% (w/v) K₂CO₃, and the combined alkaline extracts were acidified and extracted twice with equal volumes of ether. The combined ether phases were washed twice with equal volumes of water and then evaporated to dryness *in vacuo*. The residue was dissolved in 10 ml. of ether and the solution dried with anhydrous Na₂SO₄.

(b) Saliva. This was processed in a similar way to that described for rumen liquor.

(c) Blood. Blood (50 ml.) was added in a thin stream to 1 l. of chloroform-methanol (2:1, v/v). After being mixed and left overnight, it was filtered through a glass-wool pad and the filtrate reduced in volume to about 60 ml. in a rotary evaporator. The mixture was acidified and the fatty acids were extracted as described above.

(d) Urine. This was treated with 0.25 vol. of 5*N*-NaOH and kept for 16 hr. at 95° to hydrolyse any combined phenyl-substituted fatty acids. The hydrolysate was cooled and washed twice with 0.5 vol. of light petroleum (b.p. 60–80°) to remove non-saponified material. The combined petroleum phases were washed with a 0.5 vol. of water and this was added to the original water-soluble material of the hydrolysate. The combined aqueous phases were then acidified and the fatty acids extracted as described above.

Gas-liquid chromatography. Methyl esters of the fatty acids were prepared by passing excess of diazomethane through the ethereal solution. Evidence was obtained that some of the aromatic hydroxyl groups of hydroxyphenyl-substituted fatty acids were methylated. Thus two peaks were invariably seen on gas-liquid chromatography, and the size of the slower-moving component was greatly decreased by prolonged methylation (see Williams, 1962).

Gas-liquid chromatography was carried out on a Pye Argon chromatograph with a β -ionization detector. The apparatus was fitted with a capillary splitting device that diverted 98% of the gas stream from the detector. This enabled much larger columns (114 cm. $\times$ 1.2 cm. internal diam.) and column loadings (1–4 mg.) to be used and individual fatty acid esters could be recovered from the diverted gas stream. To prevent condensation of these in the capillary carrying the bulk of the gas stream, this was enclosed in an aluminium tube covered with an electrical heating element. The capillary was maintained at the same temperature as the chromatographic column by adjusting the voltage across the heater with a variable transformer. A temperature calibration was carried out initially with a fine thermocouple inserted along the capillary. The column fillings used were 10% (w/w) Apiezon L or 10% (w/w) poly(ethylene glycol adipate) on Celite (100–120 mesh; Pye).

Collection of effluent fatty acid esters. Two methods had to be used for the collection of fatty acid esters, depending on the rate at which these passed through the column. The fastest-running components, which were generally the most volatile, were absorbed by passing them through a short scrubbing column (3 cm. diam.) which was connected to the splitting capillary by silicone-rubber tubing (Fig. 1*a*). The column contained a no. 3 sintered-glass disk, *B*, and was loaded with 20 ml. of light petroleum (b.p. 40–60°) or toluene-scintillation mixture, *C*, which was maintained in position by the effluent gas stream, *A*. Measurements showed that the back pressure produced by the head of solvent caused a change in the splitting ratio so that more of the effluent gas went through the detector. Nevertheless, over 95% of the effluent gas still passed through the side capillary and so good yields of fatty acid methyl esters could be obtained.

After collection the column was inclined at an angle of 45° to the vertical, the silicone-rubber connector was withdrawn from the capillary side arm and the light petroleum solution together with washings was transferred to a flask for evaporation. It was necessary to exercise great care in

the removal of solvent as overheating or too-prolonged drying of the residue caused appreciable losses of the phenyl-substituted fatty acids. Consequently the evaporation was carried out in a rotary evaporator at 30–40° and the evaporation stopped immediately all the solvent had been removed. With longer-chain fatty acid esters and non-volatile esters in general this method of collection gave poor yields owing to the formation of solid aerosols of the ester which were not absorbed in the solvent. The method eventually adopted for the collection of these esters was a variation of the electrostatic precipitator devised by Kratz, Jacobs & Mitzner (1959). The effluent gas, *A'*, from the column was passed through a ground-glass joint connector into a glass tube (20 cm. $\times$ 0.8 cm. internal diam.) containing a central electrode, *E*, enclosed in a drawn-out glass projection (Fig. 1*b*). Tin-foil wrapped round the tube served as the second electrode, *D*. A conventional Villard power-pack circuit gave a constant potential of 3000 v superimposed on which was an alternating-current sine component giving a peak potential of 7000 v. A 22 M Ω resistor limited the current output and acted as a safety device in case of accidental contact (Fig. 1*b*).

The aerosol was precipitated on both electrodes and the methyl ester was removed from the tube by flushing through with ether or toluene scintillation mixture.

Counting techniques. Crude fatty acid mixtures extracted from biological samples and amino acid standards were assayed for radioactivity by evaporating suitable samples on planchets to form 'infinitely thin' layers. The planchets containing fatty acids were then exposed to the vapour from aq. NH_3 solution (sp.gr. 0.88) for 10 min. to convert them into their ammonium salts. These were then counted in an automatic flow counter (Nuclear-Chicago Corp.) with an efficiency of about 20% for ^{14}C .

Fig. 1. Apparatus for collection of fatty acid methyl esters from gas-liquid chromatograph. (a) Column effluent gas, *A*, is passed through a column with a sintered-glass disk, *B*, containing solvent, *C*. (b) Electrostatic precipitator and circuit diagram: *A'*, Column effluent gas; *D*, tin-foil electrode; *E*, removable wire electrode. Further details are given in the text.

Isolated fatty acid methyl esters dissolved in 7 ml. of scintillation fluid [AnalaR toluene containing 2,5-diphenyl-oxazole (4 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1 g./l.)] were counted in a liquid-scintillation counter (Panax, Redhill, Surrey) with an efficiency of 80% for ^{14}C . In recovery experiments a sample of the crude mixtures often contained material that quenched the scintillation, and consequently various dilutions were counted and the counting rates/mg. extrapolated to infinite dilution.

In some of the later experiments continuous radioactivity scans of the effluent of the gas-liquid-chromatography column were obtained by an apparatus similar to that described by James & Piper (1961). The gas effluent from the side arm was passed directly through a combustion furnace containing CuO and iron filings maintained at 700°. The gas stream was then carried by narrow bore (2 mm.) polyethylene tubing to the proportional counter which was located at a distance of 1 m. from the gas-liquid chromatograph to avoid a high background caused by the ^{90}Sr source in the β -ionization detector. Carbon dioxide (5%, by vol.) was introduced into the gas stream before it entered the counter tube to act as a quencher.

Materials. ^{14}C -labelled amino acids and algal protein were purchased from The Radiochemical Centre, Amersham, Bucks.; [^{14}C] indicates uniform labelling. The amino acids contained no radioactive material that was ether-soluble. For each experiment 5–30 μC of ^{14}C -labelled amino acid was used and it was mixed with 170 mg. of casein hydrolysate (Oxo Ltd., London). Commercial samples of the following were obtained: 3-phenylpropionic acid, phenylacetic acid and its *p*-hydroxy derivative, 3-phenyllactic acid (from L. Light and Co. Ltd., Colnbrook, Bucks.); *p*-hydroxyphenylpyruvic acid (from Sigma Chemical Co., St Louis, Mo., U.S.A.); 3-(*p*-hydroxyphenyl)propionic acid (from Calbiochem, Los Angeles, Calif., U.S.A.). 3-Phenylpyruvic acid was synthesized by the method of Herbst & Shemin (1943) and had m.p. 151–154°. 3-(*p*-Hydroxyphenyl)lactic acid samples were kindly donated by Dr I. Smith and Dr L. I. Woolf.

Conversion of ^{14}C -labelled phenyl-substituted fatty acids into derivatives. The methyl esters of these acids collected from the column were first hydrolysed at 65° for 3 hr. in 0.5N- NaOH in 50% (v/v) methanol. The free acid was then recovered by acidification of the hydrolysate and extraction with ether.

The method used for the preparation of the *p*-phenylphenacyl esters of 3-phenylpropionic acid and benzoic acid was that described by Cheronis & Entrikin (1957). The amide of phenylacetic acid was prepared by passing dry NH_3 gas through a methanolic solution of the methyl ester for 5 hr. at 0°.

RESULTS

Isolation and characterization of phenyl-substituted fatty acids in rumen liquor. When the fatty acids isolated from rumen liquor were examined by gas-liquid chromatography a main component was observed with a retention volume (relative to methyl decanoate) of 0.74 on Apiezon L and 3.9 on poly(ethylene glycol adipate) columns. Samples of the methyl ester of this acid were isolated and examined by three physical techniques: (1) infrared spectroscopy; (2) nuclear magnetic resonance

(kindly carried out by Dr D. Chapman); (3) mass spectrography (kindly carried out by Dr T. H. Shuttleworth). These suggested that the unknown acid contained an aromatic ring and less than three methylene units/molecule. Comparison of these records with those of authentic methyl 3-phenylpropionate showed the two methyl esters to be identical. Fig. 2 shows the infrared-absorption spectra of the two methyl esters measured in potassium bromide disks. Similarly the methyl 3-phenylpropionate ran to a position identical with that of the unknown compound on gas-liquid chromatography, both on Apiezon L and on poly-(ethylene glycol adipate) columns.

The logarithms of retention volumes of other minor peaks on the gas-liquid chromatogram (on Apiezon L) of the rumen-liquor fatty acids when compared with those of known standards suggested that there were other acids homologous with 3-phenylpropionic acid. Consequently the characteristics on the column of a synthetic sample of methyl phenylacetate were tested: its retention volume both on Apiezon L and on poly(ethylene glycol adipate) columns corresponded exactly to that of one of these peaks (Table 1).

In addition, small amounts of benzoic acid were detected in the sample by gas-liquid chromatography but here the retention volume on Apiezon L did not lie on the logarithmic plot of the other acids.

Concentrations of phenyl-substituted fatty acids in body fluids of the sheep. The finding of appreciable amounts of 3-phenylpropionic acid and phenylacetic acid in rumen liquor suggested that these acids might also be present in other body fluids of the sheep. Table 2 shows the recovery of phenyl-substituted fatty acids when the fatty acids from

rumen liquor, whole blood, urine and saliva were extracted and examined by gas-liquid chromatography. The concentrations were obtained by comparison of the peak areas measured by planimetry with the area of a standard amount of methyl ester injected into the column under the same conditions. However, since the injection of small quantities of fluid on to the column by a Hamilton syringe is subject to appreciable error, the values given can only be regarded as approximate.

3-Phenylpropionic acid was present in blood and saliva but at concentrations that were very much less than that in rumen liquor. Only traces of this acid were present in urine, which contained, on the other hand, appreciable amounts of benzoic acid and phenylacetic acid. The rumen liquor contained much less phenylacetic acid than 3-phenylpropionic acid and only traces of benzoic acid. This picture has been consistent in a large number of rumen-liquor samples examined.

Table 1. Gas-liquid chromatographic characteristics of relevant phenyl-substituted fatty acids

Experimental details are given in the text. Retention volumes are given relative to that of 3-phenylpropionic acid (methyl ester).

Acid (methyl ester)	Retention volume	
	On 10 % Apiezon L 150°	On 10 % poly-(ethylene glycol adipate) 178°
Benzoic acid	0.44	0.47
Phenylacetic acid	0.57	0.72
3-Phenylpropionic acid	1.00	1.00
4-Phenylbutyric acid	1.79	1.42
p-Hydroxybenzoic acid	3.09	16.91
p-Methoxybenzoic acid	1.87	2.11
p-Hydroxyphenylacetic acid	2.83	21.48
p-Methoxyphenylacetic acid	2.10	3.22
3-(p-Hydroxyphenyl)propionic acid	4.56	27.63
3-(p-Methoxyphenyl)propionic acid	3.61	4.30
Indol-3-ylacetic acid	8.92 (at 200°)	71.25

Table 2. Distribution of phenyl-substituted fatty acids in body fluids of the sheep

Sample	Approx. concn. (m-moles/l.)		
	Benzoic acid	Phenylacetic acid	3-Phenylpropionic acid
Rumen contents	0.01	0.04	0.3
Blood	0.01	0.02	0.01
Urine	7.2	2.4	Trace
Saliva	—	0.14	0.05

Fig. 2. Infrared absorption spectrum of authentic methyl 3-phenylpropionate (bottom) and methyl 3-phenylpropionate isolated from rumen liquor (top).

Degradation of phenylalanine into fatty acids in the rumen. Since previous work had suggested that 3-phenylpropionic acid was a microbiological degradation product of protein phenylalanine (Salkowski & Salkowski, 1879; Nawiasky, 1908), the conversion of L-[U-¹⁴C]phenylalanine into fatty acid

Table 3. *Conversion of ¹⁴C-labelled aromatic amino acids into ether-soluble volatile fatty acids in the artificial rumen*

Experimental details are given in the text. Each amino acid was mixed with 170 mg. of casein hydrolysate.

Amino acid	Percentage conversion		
	At 20 min.	At 60 min.	At 180 min.
L-[U- ¹⁴ C]Phenylalanine	21	31, 27	—
L-[U- ¹⁴ C]Tyrosine	9	13, 14	12, 11
D,L-Tryptophan(indol-3'-yl[3- ¹⁴ C]alanine)	—	3	—

Table 4. *Distribution of radioactivity in phenyl-substituted fatty acids after giving L-[U-¹⁴C]-phenylalanine*

Experimental details are given in the text. The amino acid was mixed with 170 mg. of casein hydrolysate.

Acid (methyl ester)	Percentage distribution of radioactivity		
	Rumen <i>in vivo</i> At 1 hr.	Artificial rumen	
		At 1 hr.	At 2 hr.
Short-chain fatty acids	0	0	} 2
Benzoic acid	1	0	
Phenylacetic acid	96	98	94
3-Phenylpropionic acid	3	2	4
Percentage recovery of activity in fractions examined	76	83	74

was studied both in the artificial and intact rumen. Table 3 shows that about 20 % of the added radioactivity had appeared in fatty acids in the artificial rumen in 20 min. and 30 % in 1 hr. Table 4 shows the distribution of radioactivity in the fatty acid fraction 1 hr. after introducing [U-¹⁴C]phenylalanine into both systems and after 2 hr. in the artificial rumen.

Almost all of the radioactivity was present in phenylacetic acid and only small amounts were present in the 3-phenylpropionic acid peak. The identification of phenylacetic acid as the dominant radioactive product was confirmed by examining the ¹⁴C-labelled fatty acid methyl esters both on Apiezon L and on poly(ethylene glycol adipate) columns. Further, some of the material of the radioactive peak was isolated and after hydrolysis the free acid was mixed with 50 mg. of authentic phenylacetic acid. The specific radioactivity did not change appreciably on recrystallization twice from light petroleum and after conversion into the crystalline amide with the correct m.p. (Table 5).

Conversion of tyrosine into fatty acids in the rumen. Similar experiments were performed with L-[U-¹⁴C]-tyrosine. With this amino acid the percentage of the radioactivity appearing in fatty acids in the artificial rumen was appreciably less, being only 9 % in 20 min. and 13–14 % in 1 hr.; after 3 hr. of incubation the percentage had not increased further (Table 3). Examination of the fatty acids by gas-liquid chromatography showed that in the intact rumen and in the artificial rumen after 3 hr. the major radioactive peak on the gas-liquid chromatogram was 3-phenylpropionic acid (Table 6). The identity of the material of the peak was confirmed by running on the two gas-liquid-chromatography columns and by the constancy of the specific radioactivity on recrystallizing the free acid and con-

Table 5. *Confirmation of identity of radioactive peaks by recrystallization and formation of derivatives*

Experimental details are given in the text. The radioactive peak from gas-liquid chromatography was hydrolysed and the free acid mixed with 50 mg. of authentic carrier.

Purification step	Specific radioactivities (counts/min./mg. of acid)		
	3-Phenylpropionic acid (isolated from artificial rumen 3 hr. after giving L-[U- ¹⁴ C]tyrosine)	Phenylacetic acid (isolated from intact rumen 1 hr. after giving L-[U- ¹⁴ C]phenylalanine)	Benzoic acid (isolated from urine after giving L-[U- ¹⁴ C]tyrosine <i>in vivo</i>)
—	2923	160	900
Two recrystallizations from light petroleum (b.p. 40–60°)	2981	182	904
Conversion into crystalline derivative	3018	182	872
	[<i>p</i> -phenylphenacyl ester m.p. 94° (uncorrected); m.p. 95° according to Cheronis & Entriikin (1957)]	[amide m.p. 157° (uncorrected); m.p. 156° according to Cheronis & Entriikin (1957)]	[<i>p</i> -phenylphenacyl ester m.p. 166° (uncorrected); m.p. 167° according to Cheronis & Entriikin (1957)]

Table 6. *Distribution of radioactivity in phenyl-substituted fatty acids after giving L-[U-¹⁴C]tyrosine*

Experimental details are given in the text. The amino acid was mixed with 170 mg. of casein hydrolysate.

Acid (methyl ester)	Percentage distribution of radioactivity				
	Rumen <i>in vivo</i>			Artificial rumen	
	At 1 hr.	At 3 hr.	At 6 hr.	At 1 hr. (perfusing)	At 3 hr. (non-perfusing)
Benzoic acid + phenylacetic acid	4	6	4	2	4
3-Phenylpropionic acid	92	77	76	65	82
Unidentified peaks after 3-phenylpropionic acid peak	4	17	20	33	14
Percentage recovery of activity in fractions examined	89	84	81	78	83
Total activity in isolated fatty acids (counts/min./200 ml. of rumen liquor)	17 060	12 320	8 580	—	—

Fig. 3. Comparison of the fatty acid pattern (A) and radioactivity (B) on gas-liquid chromatography of the fatty acids obtained by incubating L-[U-¹⁴C]tyrosine with rumen micro-organisms. Peak 1, ether peak giving spurious radioactive counts; peak 2, methyl 3-phenylpropionate; peak 3, methyl *p*-methoxyphenylacetate; peak 4, methyl 3-(*p*-methoxyphenyl)propionate; peak 5, methyl *p*-hydroxyphenylacetate; peak 6, 3-(*p*-hydroxyphenyl)propionate. The column was poly(ethylene glycol adipate), operated at 180°; *p*-hydroxyphenylacetic acid was added to the fatty acid mixture as a marker before methylation.

verting it into the *p*-phenylphenacyl derivative (Table 5). In the artificial rumen after 1 hr. of incubation there always appeared to be an appreciable amount of radioactive substances giving peaks on the gas-liquid chromatogram which appeared after the 3-phenylpropionic acid peak (Table 6).

The identity of these peaks was investigated by continuously scanning the effluent gas stream from the chromatogram for radioactivity. The pattern of radioactivity was complicated (Fig. 3, curve B), for as well as the radioactivity in the 3-phenylpropionic peak there were four other radioactive components visible on the scan and only one of these corresponded to a defined peak located by the β -ionization detector. Experiments with a large number of markers showed that two components, 3-(*p*-hydroxyphenyl)propionic acid and *p*-hydroxyphenylacetic acid, were actually present and that the hydroxyl group in the benzene ring was in each case being only partially methylated giving two

radioactive components. In Fig. 3 the typical position of these peaks on a poly(ethylene glycol adipate) column is shown and *p*-hydroxyphenylacetic acid has been added as a marker. Measurements of the percentage distribution of radioactivity after 20 min. and 60 min. of incubation with [U-¹⁴C]tyrosine in the artificial rumen were obtained by planimetry and are given in Table 7. Whereas the percentage of *p*-hydroxyphenylacetic acid increased with the longer period of incubation, that of the 3-(*p*-hydroxyphenyl)propionic acid decreased. This suggested that the latter was acting as the immediate precursor of the radioactive 3-phenylpropionic acid which was being formed. Consequently a sample of the radioactive 3-(*p*-hydroxyphenyl)propionic acid methyl ester was isolated by gas-liquid chromatography and after conversion into the free acid was fed to an artificial rumen. After 1 hr. gas-liquid chromatography of the free fatty acids isolated from the clear rumen-liquor fraction showed that approximately half had been converted into 3-phenylpropionic acid (Table 7). In further experiments radioactive *p*-hydroxyphenylacetic acid was isolated and incubated with rumen micro-organisms for 1 hr. in the artificial rumen. The majority of this was recovered unchanged but about 10% had been converted into phenylacetic acid (Table 7).

Conversion of ¹⁴C-labelled tryptophan into fatty acids in the rumen. When L-tryptophan (indol-3'-yl-[3-¹⁴C]alanine) was admixed with 170 mg. of casein hydrolysate plus 17 mg. of unlabelled DL-tryptophan and incubated in the artificial rumen for 1 hr., only 3% of the activity was recovered in the fatty acid fraction (Table 3). Examination of the fatty acid methyl esters by gas-liquid chromatography on an Apiezon L column showed that 94% of the applied radioactivity was recovered in a single small peak with none of the activity in either the phenylacetic acid or the 3-phenylpropionic acid peak. The radioactive peak had a retention volume on the column (Table 1) that was identical with that of

indol-3-ylacetic acid methyl ester. Similarly on gas-liquid chromatography on a poly(ethylene glycol adipate) column the radioactivity moved to the same position as a methyl indol-3-ylacetate marker. Authentic indol-3-ylacetic acid was recovered in quantitative yield by the extraction procedure described.

Formation of fatty acid from uniformly ^{14}C -labelled algal protein in the rumen. In a further experiment 100 μC of uniformly ^{14}C -labelled denatured algal protein was introduced into the rumen of a sheep and samples of rumen contents were withdrawn at various times. Samples were processed in the manner described above to yield a fatty acid fraction. When the distribution of radioactivity was measured after 30 min. and 4 hr. the majority of the activity (67–69 %) was present in the short-chain fatty acid methyl esters (C_4 – C_8 aliphatic), 18–20 % was present in the methyl phenylacetate peak and only 2 % in the methyl 3-phenylpropionate peak (Table 8). The ratio of the

percentage of radioactivity present in phenylacetic acid and that in 3-phenylpropionic acid did not noticeably alter with time: thus at 30 min. it was 10, at 60 min. again 10, and at 240 min. 12.

Rate of disappearance of $[\text{U-}^{14}\text{C}]$ phenylacetic acid and $[\text{U-}^{14}\text{C}]$ 3-phenylpropionic acid from the rumen in vivo. Samples (10 μC) of $[\text{U-}^{14}\text{C}]$ phenylacetic acid and $[\text{U-}^{14}\text{C}]$ 3-phenylpropionic acid were prepared by incubating L- $[\text{U-}^{14}\text{C}]$ phenylalanine and L- $[\text{U-}^{14}\text{C}]$ -tyrosine separately in the artificial rumen and then separating the fatty acid methyl esters by gas-liquid chromatography. The esters were saponified with alkali and the fatty acids extracted into ether after acidification of the hydrolysate. Experiments were then performed in which 5 μC of one of these acids was introduced into the rumen of a sheep, and the disappearance of radioactivity from the fatty acid fraction was measured. To minimize biological variation, the same sheep was used for each experiment, an appropriate interval being allowed for complete clearance of the previous dose. Fig. 4

Table 7. *Percentage distribution of radioactivity in phenyl-substituted fatty acids as determined by radioactivity scans of the gas-liquid chromatograms after the incubation of rumen micro-organisms with L- $[\text{U-}^{14}\text{C}]$ tyrosine, $[\text{U-}^{14}\text{C}]$ p-hydroxyphenylacetic acid or $[\text{U-}^{14}\text{C}]$ 3-(p-hydroxyphenyl)propionic acid*

Experimental details are given in the text. Each substrate was incubated in the artificial rumen together with 170 mg. of casein hydrolysate without perfusion except in column 2.

Substrate	Percentage distribution of radioactivity				
	L- $[\text{U-}^{14}\text{C}]$ Tyrosine		L- $[\text{U-}^{14}\text{C}]$ -Phenylalanine	$[\text{U-}^{14}\text{C}]$ -3-(p-Hydroxy-phenyl)-propionic acid	$[\text{U-}^{14}\text{C}]$ -p-Hydroxy-phenylacetic acid
	At 20 min.	At 60 min. (perfusing)	At 20 min.	At 60 min.	At 60 min.
Acid (methyl ester)					
Benzoic acid	0	2	0	4*	2*
Phenylacetic acid	1	0	96	—	10
3-Phenylpropionic acid	73	65	4	49	10
p-Hydroxyphenylacetic acid	8	27	0	—	78
3-(p-Hydroxyphenyl)propionic acid	18	6	0	47	—

* Distribution measured by solvent collection and electrostatic precipitation.

Table 8. *Distribution of radioactivity in rumen fatty acids after giving uniformly ^{14}C -labelled denatured algal protein*

Experimental details are given in the text. Uniformly ^{14}C -labelled denatured *Chlorella* protein (0.55 mg.; 100 μC) was introduced into the rumen through a fistula 0.5 hr. after feeding the sheep with 1000 g. of hay and 200 g. of oats.

	Percentage distribution of radioactivity			
	At 0.5 hr.	At 1 hr.	At 2 hr.	At 4 hr.
Total radioactivity in fatty acids isolated from 250 ml. (counts/min.)	6700	5010	4600	4050
Acid (methyl ester)				
Short-chain fatty acids				67
Benzoic acid	69			0
Phenylacetic acid	20			18
3-Phenylpropionic acid	2			2
Unidentified peaks after 3-phenylpropionic acid peak	9			13
Percentage recovery of activity in fractions examined	78			80

shows a logarithmic plot of the disappearance rate of radioactivity from the fatty acid fraction. In each experiment the values at 30 min. are higher than those expected from the logarithmic plot. The most probable explanation of this is inadequate mixing of the sample at such a short time-interval. The rumen contents are known to take at least an hour to mix thoroughly (Gray, Jones & Pilgrim, 1960), and sampling before this near to the fistula is likely to yield a higher radioactivity than might be expected from an even distribution throughout the rumen.

The rate of disappearance of radioactivity in the rumen from the $[U-^{14}C]$ phenylacetic acid experiment was slightly greater than the rate of disappearance when $[U-^{14}C]$ 3-phenylpropionic acid was administered (Fig. 4). Since, however, there was much more 3-phenylpropionic acid than phenylacetic acid in the rumen, this would mean that the rate of loss of 3-phenylpropionic acid on a weight basis would be greater than that of phenylacetic acid.

Examination of the fatty acid methyl esters, isolated from rumen samples after introducing $[U-^{14}C]$ 3-phenylpropionic acid and $[U-^{14}C]$ phenylacetic acid, by gas-liquid chromatography failed to detect any interconversion between these two acids or the formation of other fatty acids extracted by the method described above.

Fig. 4. Rate of disappearance of radioactivity from the rumen after introducing $[U-^{14}C]$ phenylacetic acid and $[U-^{14}C]$ 3-phenylpropionic acid. Approx. $5\mu C$ of radioactivity was administered through the rumen fistula at zero time. ○, 3-Phenylpropionic acid; ●, phenylacetic acid.

Urinary excretion of phenyl-substituted fatty acids after the administration of L-[U- ^{14}C]phenylalanine and [U- ^{14}C]phenylacetic acid. Collections of urine were made after the introduction of L-[U- ^{14}C]phenylalanine into the rumen, and 0.23 % of the radioactivity was voided in the fatty acid fraction in 6 hr. (Table 9). When the distribution of activity was measured by gas-liquid chromatography the major part of the activity (68 %) was localized in the methyl phenylacetate peak. If, on the other hand, the same amount of L-[U- ^{14}C]phenylalanine was injected into the circulation the excretion pattern was different. Much less (0.1 %) radioactivity was voided in the urinary fatty acids and a distribution study showed very little present in the methyl phenylacetate peak (Table 9). When [U- ^{14}C]phenylacetic acid was introduced into the rumen, urine collections over 7 hr. showed that about 1 % of the activity was excreted in the fatty acid fraction (Fig. 5), and gas-liquid chromatography revealed that the major part of this was phenylacetic acid (Table 9).

Urinary excretion of phenyl-substituted fatty acids after the administration of L-[U- ^{14}C]tyrosine and [U- ^{14}C]phenylpropionic acid. After L-[U- ^{14}C]tyrosine was introduced into the rumen it was shown that benzoic acid was the predominant radioactive component of the urinary fatty acid fraction voided up to 8 hr. (Table 10). When the rumen tyrosine metabolite, [U- ^{14}C]3-phenylpropionic acid, was introduced into the rumen about 2 % of the radioactivity was excreted in the urinary fatty acids after 7 hr. (Fig. 5), and gas-liquid chromatography showed that 43 % was present as benzoic acid. Again, a totally different result was obtained if L-[U- ^{14}C]tyrosine was injected into the jugular vein, there being almost no radioactivity in the benzoic acid appearing in the urine.

Metabolism of [U- ^{14}C]phenylacetic acid and [U- ^{14}C]3-phenylpropionic acid by sheep-liver and sheep-kidney slices. The results in the preceding section suggested that 3-phenylpropionic acid formed from tyrosine in the rumen was a precursor of urinary benzoic acid. Consequently experiments were performed in which this ^{14}C -labelled substrate isolated from an artificial rumen after giving L-[U- ^{14}C]tyrosine was incubated with slices of kidney and liver from the sheep. Similar experiments were performed with the rumen phenylalanine metabolite, phenylacetic acid. In the presence of unlabelled acetate and glucose, [U- ^{14}C]3-phenylpropionic acid was metabolized by kidney slices and to a lesser extent by liver slices (Table 11). In the kidney 2.1 % of the added radioactivity was recovered in the benzoic acid fraction isolated by gas-liquid chromatography, which indicates a conversion of $2.1 \times 9/7$, i.e. 2.7 %. The carbon dioxide recovered contained 0.63 % of the added radio-

Table 9. *Distribution of radioactivity in urinary phenyl-substituted fatty acids after the administration of L-[U-¹⁴C]phenylalanine and [U-¹⁴C]phenylacetic acid*

Experimental details are given in the text.

Acid (methyl ester)	Percentage distribution of radioactivity		
	L-[U- ¹⁴ C]Phenylalanine introduced into rumen	L-[U- ¹⁴ C]Phenylalanine injected in jugular vein	[U- ¹⁴ C]Phenylacetic acid introduced into rumen
	Urine 0-2 hr. (97 ml.)	Urine 0-4 hr. (114 ml.)	Urine 0-6 hr. (143 ml.)
Short-chain fatty acids + benzoic acid	4	5	2
Phenylacetic acid	68	7	62
3-Phenylpropionic acid	0	0	36*
Unidentified peak(s) after 3-phenylpropionic acid peak	28*	88*	
Percentage recovery of radioactivity in fractions examined	98	101	96
Total radioactivity in isolated fatty acid (counts/min./10 μ C administered)	5 355	3 220	44 800

* Collected by electrostatic precipitator.

Table 10. *Distribution of radioactivity in urinary phenyl-substituted fatty acids after the administration of L-[U-¹⁴C]tyrosine and [U-¹⁴C]3-phenylpropionic acid*

Experimental details are given in the text.

Acid (methyl ester)	Percentage distribution of radioactivity				
	L-[U- ¹⁴ C]Tyrosine introduced into rumen		L-[U- ¹⁴ C]Tyrosine injected in jugular vein		[U- ¹⁴ C]3-Phenylpropionic acid introduced into rumen
	Urine 0-2 hr. (50 ml.)	Urine 2-8 hr. (170 ml.)	Urine 0-1 hr. (50 ml.)	Urine 1-6 hr. (300 ml.)	Urine 0-7 hr. (193 ml.)
Short-chain fatty acids	0	2	0	2	0
Benzoic acid	90	72	1	4	43
Phenylacetic acid + 3-phenylpropionic acid	1	4	0	1	2
Unidentified peaks after 3-phenylpropionic acid peak	9	22	99	93	55
Percentage recovery of activity in fractions examined	71	81	91	85	95
Total radioactivity in isolated fatty acid (counts/min./10 μ C administered)	13 230	35 500	8 700	4 500	76 400

Fig. 5. Appearance of radioactivity in urinary fatty acids after the administration of [U-¹⁴C]3-phenylpropionic acid and [U-¹⁴C]phenylacetic acid into the rumen of sheep. Experimental details are given in the text. ○, 3-Phenylpropionic acid; ●, phenylacetic acid.

activity and, if it is assumed that 2 side-chain carbon atoms/molecule were oxidized, this implied a conversion of $0.63 \times 9/2$, i.e. 2.8%.

On the other hand, with liver slices the conversion of 3-phenylpropionic acid into benzoic acid was 1.8 and 0.85% for carbon dioxide. Hence with this tissue not more than one-half of the side-chain carbon atoms of the molecules converted into benzoic acid appeared as carbon dioxide.

No evidence of any appreciable oxidative metabolism of [U-¹⁴C]phenylacetic acid or conversion into other phenyl-substituted fatty acids was seen with either liver or kidney slices (Table 11).

DISCUSSION

The digestion of protein in the rumen is known to be associated with the release of free ammonia (McDonald, 1948; 1952) which presumably results from the microbiological deamination of the

Table 11. *Metabolism of [U-¹⁴C]phenylacetic acid and [U-¹⁴C]3-phenylpropionic acid by sheep-liver and sheep-kidney slices*

Tissues were transported to the Laboratory on ice and sliced free-hand. The incubation medium was Krebs-Ringer phosphate containing 1 mg. of glucose/ml., 0.3 mg. of sodium acetate/ml. and 0.5 μ C (approx. 0.5 mg.) of the sodium salt of the uniformly ¹⁴C-labelled phenyl-substituted fatty acid. Incubation was in Warburg flasks at 37° for 90 min. in O₂. Other details are given in the text.

Tissue (wet wt. of slices in mg.)	Radioactive substrate	CO ₂ recovered		Benzoic acid recovered	
		Radioactivity (counts/min.)	Percentage conversion	Radioactivity (counts/min.)	Percentage conversion*
Liver (378)	[U- ¹⁴ C]Phenylacetic acid	40	0.02	0	0
Liver (360)	[U- ¹⁴ C]3-Phenylpropionic acid	2 180	0.85	16 016	1.8
Kidney (300)	[U- ¹⁴ C]Phenylacetic acid	130	0.06	0	0
Kidney (307)	[U- ¹⁴ C]3-Phenylpropionic acid	7 230	2.8	30 744	2.7

* Percentage conversion calculated by assuming that (a) phenylacetic acid has 7 carbon atoms/molecule available for benzoic acid and 1 carbon atom/molecule for CO₂ formation; (b) 3-phenylpropionic acid has 7 carbon atoms/molecule for benzoic acid and 2 carbon atoms/molecule for CO₂ formation.

individual amino acids released by proteolysis. Lewis (1955) showed that certain amino acids such as L-aspartic, L-threonine and L-alanine were deaminated by washed sheep-rumen micro-organisms with the formation of volatile fatty acids, but he failed to find an analogous reaction with the aromatic amino acids. Similar results with mixed rumen micro-organisms of the ox have been reported by Sirotnak, Doetsch, Brown & Shaw (1953).

The present observations leave little doubt that under more physiological conditions aromatic amino acids, both in the intact and artificial rumen, can be rapidly deaminated to give phenyl-substituted fatty acids. The production of phenylacetic acid from phenylalanine suggests that the amino acid is acting as a hydrogen donor in the coupled deamination of a pair of amino acids by the Stickland (1934) mechanism (eqn. 1). Evidence for such a reaction in the rumen was obtained by El-Shazly (1952), who found that alanine and proline produce 5-aminopentanoic acid. Likewise the formation of indol-3-ylacetic acid from DL-trypto-

phan is probably the result of a similar type of reaction (eqn. 2). The formation of indol-3-ylacetic acid from tryptophan by washed bovine-rumen micro-organisms has been demonstrated by Lacoste (1961), who in addition detected 3-(indol-3'-yl)propionic acid in the incubation medium.

The breakdown of tyrosine into phenyl-substituted fatty acids seems to be more complicated. The most likely mechanism for the formation of *p*-hydroxyphenylacetic acid is again for the tyrosine to act as a donor amino acid in a coupled Stickland reaction (eqn. 3). In mammals, *p*-hydroxyphenylacetic acid is known to be a major terminal metabolite of tyramine (Ewins & Laidlow, 1910) and a minor terminal metabolite of 3-(*p*-hydroxyphenyl)-pyruvate (Schayer, 1953). However, the oxidation of such tyrosine metabolites is not likely to proceed under the anaerobic conditions prevailing in the rumen, and in additional experiments neither of these metabolites was found as an intermediary of L-[U-¹⁴C]tyrosine degradation by rumen micro-organisms.

The formation of 3-(*p*-hydroxyphenyl)propionic acid from tyrosine suggests that this might also be acting as a hydrogen acceptor in a Stickland reaction (eqn. 4). Such a dual role of tyrosine as both acceptor and donor in a Stickland reaction is not improbable because of the great diversity of microbiological types in the mixed rumen contents. Indeed the small trace of 3-phenylpropionic acid formed from phenylalanine (Table 4) might be formed by a similar mechanism. Short-term experiments with ^{14}C -labelled compounds designed to detect an intermediary in this reaction either in the rumen liquor or the organisms themselves were not successful, which suggests a tight coupling of the reaction if it does take place in stages. Once formed, the 3-(*p*-hydroxyphenyl)propionic acid is slowly converted into 3-phenylpropionic acid, probably by removal of the aromatic hydroxyl group and the formation of water. Hydrogenation of unsaturated fatty acids in the rumen is a well-known phenomenon (Garton, 1961), and Booth & Williams (1963) have shown a dehydroxylation of caffeic acid by sheep rumen liquor.

The high concentration of 3-phenylpropionic acid in rumen liquor compared with phenylacetic acid is difficult to explain if protein is the only precursor of these acids. Plant protein rarely contains more tyrosine than phenylalanine. As the yield of phenylacetic acid from phenylalanine (30 %) in the artificial rumen is greater than that of 3-phenylpropionic acid from tyrosine (12 %), one would anticipate the reverse of the concentration difference that exists. The possibility that this difference could be due to a different type of breakdown occurring when intact protein is given instead of the free amino acids is made unlikely by the results obtained after administering ^{14}C -labelled algal protein. This plant protein contains roughly equal amounts of phenylalanine and tyrosine, whose carbon atoms would be of the same specific radioactivity. Yet, at all time-intervals after the feeding, the phenylacetic acid recovered from the rumen had at least ten times more counts than did the 3-phenylpropionic acid.

A second possibility was examined, namely that 3-phenylpropionic acid in excess was entering the rumen liquor by exchange-diffusion from the blood or by recirculation via salivary secretion which is known to be appreciable in the ruminant (Annison & Lewis, 1959). However, both the blood and saliva contained appreciably more phenylacetic acid than 3-phenylpropionic acid. Further, no evidence could be obtained that phenylacetic acid was removed from or metabolized in the rumen at a faster rate than was 3-phenylpropionic acid. In fact, the contrary was true, for comparison of the rates of disappearance of radioactivity when $[\text{U-}^{14}\text{C}]$ 3-phenylpropionic acid and $[\text{U-}^{14}\text{C}]$ phenyl-

acetic acid were added to the rumen showed that a greater weight of the former is removed or metabolized in the same time. These results suggest therefore that dietary phenylalanine is a major precursor of rumen phenylacetic acid. On the other hand, dietary tyrosine appears to be only a minor precursor of rumen 3-phenylpropionic acid, with unknown food components producing the bulk of this acid found in the rumen.

The metabolism of phenylalanine and tyrosine by the rumen micro-organisms is in striking contrast with that which they undergo in mammalian tissues (Henderson *et al.* 1962). This is clearly demonstrated by the different urinary secretion pattern when they are introduced into the rumen and general circulation.

When $[\text{U-}^{14}\text{C}]$ phenylalanine is introduced into the general circulation little radioactivity appears in the excreted phenylacetic acid. However, when $[\text{U-}^{14}\text{C}]$ phenylalanine or its rumen metabolite, $[\text{U-}^{14}\text{C}]$ phenylacetic acid, is introduced into the rumen, ^{14}C -labelled phenylacetic acid is excreted in the urine. This indicates that ruminal metabolism of phenylalanine is an important source of urinary phenylacetic acid, although not necessarily the only one.

Similarly, on injection of $[\text{U-}^{14}\text{C}]$ tyrosine into the circulation no radioactivity appears in the urinary benzoic acid, whereas if $[\text{U-}^{14}\text{C}]$ tyrosine or its rumen metabolite, $[\text{U-}^{14}\text{C}]$ 3-phenylpropionic acid, is introduced into the rumen, the urinary benzoic acid becomes labelled. This indicates that the 3-phenylpropionic acid formed in the rumen is an important precursor of urinary benzoic acid although again not necessarily the only one. However, these results clearly demonstrate that the metabolism of aromatic amino acids by the rumen micro-organisms is outstandingly different from the mode of dissimulation by ruminant tissues.

The experiments in which $[\text{U-}^{14}\text{C}]$ 3-phenylpropionic acid and $[\text{U-}^{14}\text{C}]$ phenylacetic acid were incubated with liver and kidney slices from the sheep show that only $[\text{U-}^{14}\text{C}]$ 3-phenylpropionic acid is metabolized to an appreciable extent, radioactive benzoic acid and carbon dioxide being produced. The most probable pathway of degradation of 3-phenylpropionic acid to benzoic acid would be by losing a C_2 fragment in the fatty acid-oxidation cycle (Lynen & Ochoa, 1953; Green, 1954). Although in mammalian tissues propionic acid is not metabolized by the fatty acid-oxidation cycle (Grafflin & Green, 1948), it is likely that the introduction of the hydrophobic aromatic nucleus into the molecule allows it to be oxidized in the same way as a longer-chain aliphatic acid. The acid would first be activated to form 3-phenylpropionyl-CoA and this would be converted into benzoic acid and acetyl-CoA by one 'turn' of the cycle. The acetyl-

CoA would then be oxidized to carbon dioxide through the tricarboxylic acid cycle. With the kidney slices the yield of radioactivity in the carbon dioxide released is quantitative, which suggests that both carbon atoms are completely oxidized. The much lower yield of radioactive carbon dioxide with the liver slices could indicate that part of the acetyl-CoA radioactive carbon atoms are being removed from the cycle via side reactions such as the conversion of α -oxoglutarate into glutamic acid (see Weinman, Strisower & Chaikoff, 1957). It is known that [1- ^{14}C]acetate used as a substrate for sheep-liver slices gives a much greater yield of radioactive carbon dioxide than does [2- ^{14}C]acetate (Leng & Annison, 1963).

SUMMARY

1. 3-Phenylpropionic acid has been isolated from sheep rumen liquor and characterized by gas-liquid chromatography, infrared spectroscopy, nuclear magnetic resonance and mass spectrography.

2. Smaller amounts of phenylacetic acid and benzoic acid have been identified in the same fluid by gas-liquid chromatography.

3. The concentration of all three phenyl-substituted fatty acids has been measured in the blood, rumen liquor, saliva and urine of the sheep.

4. Uniformly ^{14}C -labelled L-phenylalanine is converted into radioactive phenylacetic acid by rumen micro-organisms. In the artificial rumen the yield is 30%.

5. In the same system uniformly ^{14}C -labelled L-tyrosine is converted into radioactive fatty acid with a yield of 12–15%. 3-Phenylpropionic acid is the predominant product but *p*-hydroxyphenylacetic acid and 3-(*p*-hydroxyphenyl)propionic acid can also be detected.

6. Uniformly ^{14}C -labelled 3-(*p*-hydroxyphenyl)propionic acid is converted into ^{14}C -labelled 3-phenylpropionic acid by rumen micro-organisms, whereas there is much less conversion of uniformly ^{14}C -labelled *p*-hydroxyphenylacetic acid into ^{14}C -labelled phenylacetic acid.

7. In the artificial rumen small yields of ^{14}C -labelled indol-3-ylacetic acid are obtained from tryptophan (indol-3'-yl[3- ^{14}C]alanine).

8. Uniformly ^{14}C -labelled algal protein introduced into the intact rumen is converted into radioactive volatile fatty acids, including phenylacetic acid and a little 3-phenylpropionic acid.

9. The rate of disappearance of ^{14}C -labelled 3-phenylpropionic acid and phenylacetic acid from the intact rumen has been measured.

10. On introducing uniformly ^{14}C -labelled L-phenylalanine or uniformly ^{14}C -labelled phenylacetic acid into the rumen, radioactive phenyl-

acetic acid is excreted in the urine. Very little is excreted on injecting uniformly ^{14}C -labelled L-phenylalanine into the circulation.

11. When uniformly ^{14}C -labelled L-tyrosine or uniformly ^{14}C -labelled 3-phenylpropionic acid is added to the rumen, radioactive benzoic acid is excreted in the urine. None is found in the urine on injecting uniformly ^{14}C -labelled L-tyrosine into the circulation.

12. Slices of liver and kidney from the sheep metabolize uniformly ^{14}C -labelled 3-phenylpropionic acid forming radioactive benzoic acid and carbon dioxide. No metabolism of uniformly ^{14}C -labelled phenylacetic acid could be detected.

13. The significance of these results is discussed. It is concluded that dietary phenylalanine is the main precursor of rumen phenylacetic acid and one of the precursors of this acid in the urine. Dietary tyrosine is a minor precursor of rumen 3-phenylpropionic acid and one of the precursors of urinary benzoic acid.

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Acetate Utilization and the Turnover of Citric Acid-Cycle Components in Pregnant Sheep

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Acetate is an important metabolic substrate in the ruminant. It is the major product of ruminal fermentation and is readily absorbed by the ruminal epithelium and transferred to the blood stream. A number of workers (Jarrett, Potter & Filsell, 1952; Pugh & Scarisbrick, 1952; Reid, 1958) have studied the rate of fall of plasma acetate concentration after intravenous administration, but it is difficult to assess the significance of utilization rates obtained when plasma concentration is high and is falling rapidly. Annison & Lindsay (1961) studied acetate utilization in non-pregnant sheep by a constant-infusion isotope-dilution technique similar to the one used by Steele, Wall, de Bodo & Altszuler (1956) for the measurement of glucose-utilization rates in dogs. Annison & Lindsay obtained rates varying from 0.7 to 5.5 mg./min./kg. in animals that had plasma acetate concentrations of 0.25–1.09 m-moles/l. Essig, Norton & Johnson (1961), using a similar technique, obtained rates of 9.2 and 12.0 mg./min./kg. in two castrated male sheep 3–4 hr. after feeding.

Clearly there is a considerable range of acetate-utilization rates in non-pregnant sheep. Increased utilization rates might be expected in pregnant animals if rapidly growing foetal tissues utilize acetate at a greater rate than maternal tissues. It was decided to measure acetate-utilization rates in early and late pregnancy in normal sheep and in late pregnancy in under-fed sheep by the continuous-infusion isotope-dilution method. In the under-fed

animals, the amount of carbohydrate synthesized from propionate is believed to be low, and overall acetate-utilization rate might well be increased as a result of the mobilization of depot fat to satisfy energy requirements. In most animal species the administration of [$1\text{-}^{14}\text{C}$]acetate gives rise to a higher rate of labelling of expired carbon dioxide than does the administration of [$2\text{-}^{14}\text{C}$]acetate. Weinman, Strisower & Chaikoff (1957) suggested that the carbon dioxide ratio, i.e. the ratio of the specific activities of expired carbon dioxide when [$1\text{-}^{14}\text{C}$] and [$2\text{-}^{14}\text{C}$]acetate respectively are given, is an indication of the turnover of citric acid-cycle components. These carbon dioxide ratios have been determined in non-pregnant sheep and in well-fed and under-fed pregnant sheep.

MATERIALS AND METHODS

Experimental animals. Clun Forest ewes were used throughout. When the presence of a normal oestrus cycle had been confirmed by the use of a vasectomized ram, they were run with a normal ram until two oestrus cycles had been missed. Two series of experiments were performed (1958–1959) and (1959–1960). Pregnancy was confirmed radiographically about 4 weeks before expected parturition. Animals were maintained in paddocks until required for experiments, and were allowed to graze freely. In the last 2 months of pregnancy, they were also given concentrates (a mixture of rolled oats, linseed cake, flaked maize and bean meal) sufficient to permit a small but steady increase in weight throughout the period. Under-fed animals (1958–1959) received no concentrates for 6 days before experiment, but grazed freely on what little grass was available on an almost bare pasture. Partially starved

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