The Fermentation of Pentoses and Uronic Acids by Bacteria from the Rumen Contents of Sheep

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Although the fermentation of cellulose in the rumen of sheep and cattle has received considerable attention (Vaartiovaara, Suomalainen & Arhimo, 1944; Elsden, 1945; Marston, 1948; Sijpesteijn, 1948; Hungate, 1950), comparatively little work has so far been carried out on the almost equally important hemicellulose fermentations which occur when grass, straw and hay is fed to the animal (cf. McAnally, 1942). This is most certainly owing to the difficulties of obtaining sufficient quantities of a purified substrate which has not been degraded during chemical treatment.

The hemicelluloses occurring in greatest quantity in natural fodders are those composed mainly of xylose and uronic acids, with arabinose and galactose occurring to a lesser extent (Norman, 1937; Weihe & Phillips, 1940; Whistler, 1951). It has been demonstrated that in the ruminant, approximately 50% of the pentosans of the feed are utilized (McCollum & Brannon, 1909; Fraps, 1930) and that approximately 30–40% is utilized in the rumen (Marshall, 1949), and since pentosans may account for 20–25% of the dry matter of the feed (Fraps, 1930) the fermentative breakdown of these substances assumes considerable importance. Further, since pentose sugars are probably not readily utilized by the animal body, they will be of greater value to the ruminant if they are converted to other products by microbial fermentation.

As a preliminary to a study of pentosan fermentation it was considered that the study of the fermentation of the readily available xylose would be of value and isolations of xylose-fermenting organisms from the rumen of sheep were attempted, under conditions believed to be not too widely different from those in the rumen.

The liquid in the rumen consists of sheep saliva diluted to some extent with plant juices and water consumed. The main form of nitrogen is ammonia, but small quantities of organic nitrogen are also present. In addition rumen liquor contains growth factors synthesized by the micro-organisms. It is neutralized by the acidic products of fermentation, chiefly volatile fatty acids and carbon dioxide. The composition of sheep saliva as determined by McDougall (1948) shows it to be an alkaline bicarbonate and phosphate buffer. This formed the

basis for the salt medium used by Elsden (1946) to study '*in vitro*' fermentations of rumen micro-organisms. A similar medium has been employed in this study, suitably modified to enable pure culture isolations to be carried out.

METHODS

Experimental animals and withdrawal of rumen liquor.

The sheep used were two 1-year old Cheviot wethers fitted with ruminal and duodenal cannulae (Phillipson & Innes, 1939; Phillipson, 1948), and were maintained on a meadow-hay diet. The hay, of which a large stock had been set aside, was fed to the sheep twice daily at 12 hr. intervals and the food and water were removed 2 hr. after feeding. This practice prevents any changes occurring in the rumen during the next 10 hr. owing to a fresh influx of food and water. Samples were withdrawn through the rumen cannula by means of a wide-bore glass tube, 2 hr. after feeding, and were strained through six layers of surgical gauze before use.

Preparation of media

Liquid salt basal medium reproducing rumen conditions.

The basal acetate/salt medium had the following composition: 0.15M-KCl 36 ml., 0.11M-CaCl₂ 8 ml., 0.15M-KH₂PO₄ 8 ml., 0.15M-MgSO₄ 8 ml., 0.15M-(NH₄)₂HPO₄ 40 ml., acetic acid (glacial) 6.3 ml., water to 900 ml. Sterilized at 120° for 20 min. Sodium carbonate (12.6 g.) was sterilized dry, in a separate container.

The sterile carbonate, when added to the sterile salt medium, produced a medium at pH 6.8 buffered with bicarbonate and acetate and saturated with CO₂. The supplement of rumen liquor was then added.

The basal phosphate salt medium was prepared as follows. Double strength basal salt medium (450 ml.) in which the acetic acid had been replaced by 1 ml. of syrupy phosphoric acid was sterilized at 120° for 20 min. 450 ml. of 3% (w/v) NaHCO₃ was sterilized by Seitz filtration under a slight vacuum and added to the salt medium. If necessary, a final adjustment of the pH to 6.8–7.0 was made with sterile syrupy phosphoric acid. Cleared and sterilized rumen liquor supplement (100 ml.) was then added.

Sterilization of supplement containing growth factors and organic nitrogen. The gauze-strained rumen samples were centrifuged at 2500 g for 1 hr. and the pH of the supernatants was adjusted to 6.8 with glacial acetic acid. The liquid was centrifuged at 25,000 g for 15 min. and the almost clear supernatants were passed through a Seitz filter and stored at 5°. Under these conditions there was no change in the pH owing to loss of CO₂ during manipulation.

The supplement added to the phosphate salt medium did not contain additional acetic acid and was prepared as rapidly as possible to prevent alkalinity developing.

The calculated ionic composition of the isolation medium is shown in Table 1.

Table 1. *Composition of the basal medium used for the isolation of xylose-fermenting bacteria*

Ion	Concentration (calc.) (mg./100 ml.)	Concentration in sheep saliva (McDougall, 1948) (mg./100 ml.)
Na	493	370-462
K	39	16-46
Ca	5	1.6-3.0
Mg	6	0.6-1.0
NH ₄	20 (as N)	9-20 (as N)
SO ₄	10.5	—
Cl	20	25-43
P	36	37-72
HCO ₃	1000	440
AcO	100	—

Sterile rumen liquor 10-15% (v/v).

Preparation of solid medium. This was prepared by mixing, at 50°, equal volumes of 4% (w/v) neutral agar-agar (Baird and Tatlock Ltd.) containing 0.05M-K₂HPO₄, and the complete basal medium containing the carbohydrate supplement, and then pouring into Petri dishes and cooling rapidly in the refrigerator. Under these conditions, no loss of CO₂ occurs sufficient to cause the plates to become alkaline within 20 min. As an additional check on the process, a few drops of bromothymol blue indicator were incorporated into some of each batch of plates. If the indicator became blue the batch was discarded.

Fermentations

Mixed rumen micro-organisms. The apparatus consisted of Büchner flasks, fitted with rubber stoppers containing an inlet capillary and carrying Bunsen valves on the side arm (cf. Elsdén, 1946). These were warmed to 40° before use.

The medium, also at 40°, was Elsdén's (1946) buffer without modification, containing 14 g. NaHCO₃/l. and saturated with CO₂ prior to use. Under these conditions the initial pH was 7.1-7.3.

Inoculum. A sample of rumen contents was collected, strained through gauze as described above, and added to the medium to bring the concentration to 20% (v/v). The sugars were added to the medium prior to inoculation. After the addition of the inoculum the contents of the flasks were gassed with CO₂. This procedure was carried out whenever a sample was removed from the flasks during fermentation. Incubations were for 18-20 hr. at 40°, the flasks being shaken every half-hour for the first 8-10 hr. The fermentations became very vigorous in 2-4 hr. Since the initial concentration of NaHCO₃ was high, the pH did not fall below 6.6-6.8 during the fermentation.

Analytical methods

Carbohydrates were estimated in the fermentation solutions by the method of Hagedorn & Jensen (1923) after removal of the protein by ZnSO₄ and NaOH (Neish, 1946).

Standard calibration curves were prepared for each carbohydrate.

Volatile acid. Total volatile acids were determined by distillation of samples in the Markham still (Markham, 1942). The fermentation liquid (10 ml.) was pipetted into the still followed by 8 g. of MgSO₄·7H₂O, 5 ml. of 10% (w/v) sodium tungstate and two drops of *n*-octanol; 4 ml. of 10N-H₂SO₄ were rapidly added and the stopper was inserted in the still. Each 50 ml. of distillate was titrated in a stream of CO₂-free air with carbonate-free 0.05N-NaOH until the titration reached the blank value, phenol red being the indicator.

This procedure enabled the presence of lactic acid to be detected, since all the volatile acids higher than acetic distilled in the first three fractions. Formic acid was completely distilled in five fractions. If lactic acid was present, no more than seven fractions were collected. In these instances the titrations were 0.1-0.2 ml. above the blank.

The bulked fractions were distilled with HgO and MgSO₄ (Friedemann, 1938) to remove formic and lactic acid. The distillates were titrated with NaOH and evaporated to dryness, the final evaporation being carried out in a micro-Kjeldahl flask, and the acids were extracted from the sodium salts as described by Elsdén (1946).

The individual acids in the extract were fractionated and estimated on buffered silica columns (Moyle, Baldwin & Scarisbrick, 1948). By this procedure, acetic, propionic and *n*-butyric acids in a mixture were distilled and fractionated with the average recoveries shown in Table 2.

Table 2. *Recovery of fatty acids after distillation and analysis by chromatography (Moyle et al. 1948)*

Acid (and source)	Recoveries (%)	
	Mean	Range
Acetic acid (A.R. Na salt)	99.5	87-113
Propionic acid (from recrystallized propionamide)	89.7	82-94
Butyric acid (B.D.H. reagent Na salt)	92.3	89-95

Formic acid. Estimated by distillation of a fresh sample of fermentation liquid and bromine oxidation of the concentrated distillate (Neish, 1946).

Lactic acid. Determined by the method of Friedemann & Graesser (1933) in the fermentation liquid cleared of protein by Zn(OH)₂ and treated with copper/lime (Van Slyke, 1917) to remove residual carbohydrates and acetoin.

Ethanol. The volatile neutral fraction calculated as ethanol was determined in two ways. On a large sample, 10 ml. of the neutral, cleared, solution and 10 g. of NaCl were steam distilled in the Markham still and the first 50 ml. containing the ethanol were collected. Samples were estimated for volatile neutral substances by dichromate oxidation (see Neish, 1946). On a small scale the method of Winnick (1942) was used.

Succinic acid. By the method of Krebs (1937), using sheep heart succinoxidase.

2,3-Butanediol and acetoin. These were not differentiated, but were estimated as total periodate-oxidizable substances by the method of Neish (1946) and calculated as butanediol.

Sugars

D-(+)-Xylose and D-(+)-cellobiose were bacteriological sugars obtained from Thos. Kerfoot. L-(+)-Arabinose was from L. Light and Co., D-(+)-galacturonic acid from Roche Products Ltd. D-Glucuronolactone (supplied by the courtesy of Corn Products Ltd., New York), was given by Dr G. A. Levvy. The lactone was converted to the sodium salt by electrometric titration to pH 8.0 with caustic soda. D-(+)-Glucose was British Drug Houses 'Analar' anhydrous dextrose.

The isolation of xylose-fermenting organisms

Inoculations from *in vitro* xylose fermentations, carried on for 24 hr. only, were made into a sterile basal medium containing 0.4% xylose (w/v) contained in sterile 50 ml. conical flasks fitted with rubber stoppers and a Bunsen valve. The flasks were incubated for 18–24 hr. at 40°. Streak plates were made from the second or third enrichment cultures and were incubated under an atmosphere of 95% N₂ and 5% CO₂ at 40°.

Colonies were picked into xylose basal medium, and the subsequent growth was again streaked on agar. An isolate was not considered pure unless two such passages through liquid and solid medium yielded identical homogeneous colonies which appeared to consist of morphologically similar organisms. The cultures were stored in slabs in basal+agar medium at 2°. No organism was considered worth studying unless four loops of a 24 hr. culture inoculated in 100 ml. of basal medium completely fermented 0.4% xylose in 12–18 hr.

RESULTS

Fermentations with mixed rumen micro-organisms

The volatile fatty acids formed by the fermentation of xylose and glucuronic acid are presented in Tables 3 and 5, which show that acetic acid predominated. Lactic acid was absent in both fermentations. Microscopic examination of the fermentation liquids showed that a large number of small rod-like organisms were present, and from these xylose enrichment cultures the organisms described below were isolated.

Very little volatile acid was produced in the absence of added substrate. The increases were from 0.4 to 0.6 mmol. total volatile acids per 100 ml. fermentation liquid in 20 hr., and of this acetic acid accounted for approximately 50% and propionic and butyric acids for almost equal quantities of the remainder. The quantities were too small to be accurately estimated by the methods used.

Pure cultures

Seventeen strains were isolated and five of these were studied in more detail. These five were Gram-negative rods with slightly rounded ends, 1 μ . wide and 3–5 μ . long. On agar in 24 hr., circular colonies 1.5–2.00 mm. wide were formed which showed a

Table 3 (a). *The products of fermentation of xylose by mixed micro-organisms from the rumen of sheep fed on hay*

(The carbohydrate was completely fermented in each case. Values in mmol. and in mg. atoms carbon.)

Exp. no.	1		2		3		4		5	
	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon
Product										
Acetic acid*	1.63	3.26	1.41	2.82	2.62	5.24	2.5	5.0	1.90	3.80
Propionic acid*	0.85	2.55	1.24	3.72	0.42	1.26	0.50	1.50	0.51	1.52
Butyric acid†	0.31	1.24	0.30	1.20	0.71	2.84	0.65	2.60	0.57	2.28
Formic acid	0.39	0.39	0.34	0.34	0.80	0.80	0.10	0.10	0.10	0.10
Lactic acid	0	0	0	0	—	—	—	—	—	—
Total carbon	—	7.44	—	8.08	—	10.14	—	9.20	—	7.70
Total volatile fatty acids	2.78	7.05	2.95	7.74	3.75	9.34	3.15	9.10	2.98	7.60
Xylose added	4.01	20.05	4.27	21.35	3.62	18.10	3.62	18.10	3.62	18.10

* All values are corrected for the original content of the acids in the added rumen liquid.

† Butyric acid, as estimated by the method of Moyle *et al.* (1948), may contain isobutyric acid.

Table 3 (b). *The percentage of the carbon of xylose fermented by mixed rumen micro-organisms found in the acetic and propionic acids produced, and the molar ratios of the products formed to each other and to the substrate fermented*

(Calculated from the data in Table 3(a))

Exp.	Total carbon recovered (%)	Ratios (mmol.)		Carbon recovered	
		Volatile acid		As acetic acid	
		Substrate used	Acetic/Propionic	(%)	As propionic acid (%)
1	36.2	0.70	1.63	16.0	12.4
2	37.8	0.69	1.12	13.2	17.4
3	55.8	1.0	6.5	29.0	7.0
4	50.8	0.87	5.0	27.6	8.3
5	42.5	0.71	3.8	21.0	8.4

Table 4. Fermentation reactions given by cultures isolated from xylose-enrichment cultures of mixed rumen micro-organisms

(A.G., Acid + Gas; 0, no reaction; -, not tested; V.P., Voges-Proskauer reaction. All cultures grew on peptone-NaCl without further additions.)

Culture	V.P. reaction	Gelatin stab	Citrate	Xylose	Glucose	Lactose	Glycerol	Sorbose	Mannose	Raffinose	Maltose	Sucrose	Dulcitol	Mannitol
1	+	0	0	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.
14	+	0	0	A.G.	A.G.	A.G.	A.	0	A.G.	A.	A.	0	A.	A.G.
15	+	0	0	A.G.	A.G.	A.G.	A.	0	A.G.	A.	A.	0	A.G.	A.G.
16	+	0	0	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	A.G.	A.	A.	A.G.
17	+	0	-	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	-	-	-	-

greenish opalescence in transmitted light. The organisms were non-motile, facultative anaerobes.

In Table 4 are shown the distinctive fermentation reactions of these organisms, together with some characterizing reactions. The organisms appear to correspond to intermediate strains of *Escherichia coli*, but differ mainly in the ability to produce small quantities of acetoin and to ferment cellobiose. The non-utilization of citrate indicates that the organisms are of the intestinal type, rather than soil or water organisms (Topley & Wilson, 1946).

Fermentations with pure cultures of the organisms

The pure strains were inoculated into the phosphate basal medium containing the carbohydrates known to be components of hemicelluloses, and after incubation for 24 hr. the cultures were analysed for volatile acids, ethanol, and lactic acid and periodate-oxidizable substances. The fermentation liquids of culture 14 were also analysed for succinic acid. The results are presented in Table 6.

DISCUSSION

It has been found possible to prepare, in a relatively simple way, both liquid and solid media which resemble the rumen liquid of animals fed on hay. The importance of obtaining such conditions has been stressed by a few workers. Hungate (1942) showed that the osmotic pressure of synthetic media must be identical with that of the rumen liquid in order to culture certain rumen protozoa. Vaartiovaara *et al.* (1944) stressed the importance of bicarbonate buffers in order to reproduce the conditions necessary for the rapid fermentation of cellulose by rumen enrichment cultures, while Elsdén (1946), Sijpesteijn (1948) and Hungate (1950) also used bicarbonate-buffered medium in their *in vitro* studies. Only Elsdén (1946), however, appears to have paid attention to the high salt concentrations also necessary.

The sterilization of the rumen-liquid supplement which has been added to such media has received less attention. Hungate (1950) autoclaved rumen liquid under a carbon dioxide atmosphere, while Sijpesteijn (1948), recognizing that the liquid became alkaline on heating, removed excess bicarbonate by means of calcium chloride, before sterilization by heat. Since the composition of rumen liquid is unknown as regards growth factors, it seems more desirable to sterilize by Seitz filtration and to adjust the pH with an acid naturally occurring in the rumen, in order to prevent alkalinity developing.

Such media are low in organic nitrogen, allowing the carbohydrate source to be the major selective factor in *in vitro* studies, thus decreasing the possible isolation of artifacts and of fermentation products

Table 5. *The products of the fermentation of glucuronolactone by mixed rumen micro-organisms*

(The carbohydrate was completely fermented in each case.)

Product	Exp. 1			Exp. 2		
	mmol.	mg. atoms carbon	Total carbon (%)	mmol.	mg. atoms carbon	Total carbon (%)
Acetic acid	2.36	4.72	37.4	1.70	3.40	27.0
Propionic acid	0.23	0.69	5.4	0.36	1.08	8.6
Butyric acid	0.19	0.76	6.0	0.08	0.32	2.5
Total carbon recovered	—	6.17	—	—	4.80	—
Glucuronic acid used	2.1	12.6	—	2.1	12.6	—
% Carbon recovered	—	—	48.8	—	—	38.1
Ratio: $\frac{\text{Acetic}}{\text{Propionic}}$	10.0	6.9	—	4.8	3.1	—

Table 6. *Products of fermentation of carbohydrates by five strains of coliform organisms isolated from enrichment cultures of rumen micro-organisms*

(No propionic acid was produced in any fermentation. The carbohydrates were completely fermented in all cases. Values in mmols.)

Product	Strain 1				Strain 14			
	Xylose	Glucuronic acid	Glucose	Cellobiose	Xylose	Glucuronic acid	Glucose	Cellobiose
	Strain 1				Strain 14			
Formic acid	0.84	0.69	1.36	3.08	0.87	1.31	1.36	1.66
Acetic acid	0.71	1.13	0.92	0.94	0.88	1.09	1.40	0.90
Lactic acid	0	0	0.95	0.23	0	0.06	0.55	0.23
Succinic acid	0	—	—	—	0	0	0	0
Ethanol	0	0	0.56	0	—	0	0.51	0
Acetoin and butane-2:3-diol	0	0	0.54	0.16	0	0	0.20	0.15
Substrate fermented	1.75	1.1	2.5	1.68*	1.75	1.1	2.5	1.68*
Product	Strain 15				Strain 16			
	Xylose	Glucuronic acid	Glucose	Cellobiose	Xylose	Glucuronic acid	Glucose	Cellobiose
	Strain 15				Strain 16			
Formic acid	1.00	0.66	1.85	N.U.	0.57	0.75	1.85	1.44
Acetic acid	0.71	1.03	1.37	N.U.	0.82	0.75	0.90	1.14
Lactic acid	0	0	0.48	N.U.	0	0	0.83	0.17
Succinic acid	0	—	—	N.U.	0	—	—	—
Ethanol	0.20	0	0.62	N.U.	0.35	0	0.61	0
Acetoin and butane-2:3-diol	0	0	0.20	N.U.	0	0	—	0
Substrate fermented	1.75	1.1	2.5	N.U.	1.75	1.1	2.5	1.68*
Product	Strain 17							
	Xylose	Glucuronic acid	Glucose	Cellobiose				
	Strain 17							
Formic acid	0.71	0.94	1.83	1.34				
Acetic acid	1.09	0.91	0.77	0.94				
Lactic acid	0	0.08	0.95	0.23				
Succinic acid	0	—	—	—				
Ethanol	0.44	0	0.08	0				
Acetoin and butane-2:3-diol	0	0	0.26	0.16				
Substrate fermented	1.75	1.1	1.75	1.68*				

* Calculated as glucose. N.U., not utilized.

derived from the organic nitrogen source (cf. Dubos & Miller, 1937). Under such conditions it may be tentatively assumed that the organisms isolated carry out degradations by metabolic pathways not entirely dissimilar to those that they would utilize during growth within the rumen. This assumption cannot yet be tested and is made with considerable reserve.

The products of fermentation of xylose by mixed rumen micro-organisms (Table 3) clearly indicate the predominance of acetic acid which accounts for an average of 21.3 % of the total carbon fermented,

while propionic acid accounts for an average of 10.7 %. The most striking feature of Table 6 is the marked difference in the products of fermentation of xylose and glucuronic acid on the one hand, and glucose and cellobiose on the other. Lactic acid, acetoin and 2:3-butanediol are not formed from xylose and glucuronic acid, but are formed from glucose and cellobiose. No volatile alcohol is produced from cellobiose or glucuronic acid, while only three strains produced volatile alcohol from xylose.

These results are distinctly different from those of Stanier & Adams (1944), Adams & Stanier (1945),

Breden & Fulmer (1931), and Reynolds & Werkman (1937), in which the fermentation of hexoses and pentoses by members of the Enterobacteriaceae yielded substantially the same products from both types of substrate. The present view of the metabolism of the hexoses by coliform organisms involves at one stage the formation of pyruvic acid as an intermediate (Stephenson, 1949). The distinction between the fermentation end products of the xylose and glucuronic acid on the one hand, and glucose and cellobiose on the other, would therefore suggest that, in the case of the first two carbohydrates, either pyruvic acid is not formed or else the mechanisms involved in the further formations of both lactic acid and acetyl methyl carbinol are not operative; for example, there may be some necessary intermediate product involved which is formed only during the fermentation of glucose and cellobiose. It is clear, however, that the fermentation of the hexuronic acid is not comparable with that of the homologous hexose, and the relationship, if any, between the metabolism of glucuronic acid and xylose is being investigated.

SUMMARY

1. A procedure is described for the preparation of solid and liquid media which closely reproduce conditions in liquid from the rumen of sheep.

2. Using these media seventeen strains of Gram-negative coliform rods of an intermediate type have been isolated in pure culture.

3. The products of fermentation (formic acid, acetic acid, lactic acid, ethanol, butyleneglycol and acetoin) formed by five of these strains from xylose, glucuronic acid, glucose and cellobiose, show marked differences both as regards the substrate and the strains of organism used.

4. The fermentation of xylose and glucuronic acid appears to follow a pathway different from that followed in the fermentation of glucose.

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