A Micro-Artificial Rumen for Isotopic Experiments

By R. M. C. DAWSON, P. F. V. WARD and T. W. SCOTT
Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 24 *June* 1963)

An artificial rumen for the study of nutrient digestion by the micro-organisms of the rumen was initially developed by Louw, Williams & Maynard (1949). In this, the micro-organisms were contained in a semi-permeable dialysis sac and the diffusible products of digestion removed by a salt solution, the composition of which approximated to that of saliva and which flowed continuously past the outside of the sac. The properties of this type of artificial rumen have been studied by Warner (1956) and Davey, Cheeseman & Briggs (1960), who suggested criteria for establishing its validity as a functioning system. Warner showed that saline of ionic composition similar to that of rumen liquor gave better results than one equivalent to saliva.

Elsden (1952) criticized the use of saline-diluted rumen liquor as a growth medium for rumen micro-organisms on the grounds that the population found at the end of the incubation was qualitatively different from that of the original rumen contents. In this respect it is generally believed that many rumen micro-organisms require factors for satisfactory growth which are present in rumen liquor and which, up to now, have been inadequately characterized (Bryant, 1959; Bryant & Robinson, 1961).

In the present design these criticisms have been largely nullified by continually perfusing with clear rumen liquor from the same unfed animal. In this way growth factors are maintained in physiological concentrations and soluble, possibly toxic, digestive products never accumulate above a concentration occurring in the rumen liquor. The apparatus is small enough to allow experiments with valuable isotopically labelled compounds. Experiments have been performed with sheep to test the biochemical validity of the artificial rumen and the results compare favourably with the properties of the intact rumen.

EXPERIMENTAL

Samples. Rumen contents (0·8–2 l.) were obtained from Clun Forest sheep with rumen fistulae. The animals were given a constant daily ration of 1000 g. of hay chaff plus 200 g. of oats. Before experiments, the animals were deprived of food overnight to decrease the amount of intrinsic foodstuff and the content of soluble digestive products. The samples were withdrawn with the aid of gentle suction from an electrical pump and collected in a Buchner flask immersed in water at 39° contained in a large vacuum-jacketed container. The contents were taken immediately to the laboratory and strained through muslin to remove coarser particles. A portion (60 ml.) was reserved for the artificial rumen and gassed at 39° with the gas mixture described below. The remainder was centrifuged at 12 500 $g_{av.}$ for 30 min. in a Servall centrifuge, and the almost clear supernatant collected for perfusion. Examination under the microscope showed it to contain a few very small bacteria.

Apparatus. The apparatus is diagrammatically illustrated in Fig. 1 and is easily constructed of glass tubes, rubber bungs and 4 mm. external diam. polythene tubing. The rumen contents were introduced into a tied-off dialysis sac, *A*, held on a glass tube (3·2 cm. diam.) by a rubber band. This tube contained an orifice, *B*, through which food could be introduced and samples withdrawn for analysis. The dialysis sac was surrounded by a larger glass tube, *C* (diam. 4·6 cm. at the bottom, 6·0 cm. at the top), which contained the clear perfusion liquid (100 ml.). The whole apparatus was immersed in a thermostatically controlled water bath maintained at 39°. The clear rumen liquor was fed from a reservoir, *D*, the rate being adjusted to about 40 ml./hr. with a screw clip. A gas mixture [N_2–CO_2–CH_4 (19:11:10, by vol.) or N_2–CO_2 (19:1, v/v)] was presaturated with water vapour at 39° and used to gas and stir the contents of the dialysis sac. It was then passed through a tube, *E*, to stir and gas the perfusion liquor. It finally lifted the excess of perfusion liquor through the exit tube, *F*, for collection in a standard fraction collector. An anti-splashing device consisting of a wider-bore glass tube was used at the end of this exit tube. For trouble-free working it was advantageous to have the level of the fraction collector below the level of the artificial rumen to prevent the development of too great a pressure head. Although not essential, in later experiments a pre-gassing chamber, *G*, was introduced into the perfusion feed pipe. The artificial rumen was fed through the orifice *B* with the hay-chaff diet ground to a fine powder in an attrition mill (model 9A; Lee Engineering Co., Milwaukee, Wis., U.S.A.). This operation was facilitated by introducing the weighed food sample into the end of a suitable glass tube where it was maintained in position by moistening the tip with rumen liquor. It was then inserted through the orifice and the contents were expressed into the dialysis sac with a closely-fitting long glass rod inserted into the tube and acting as a plunger.

Before samples were withdrawn the micro-organisms were vigorously gassed for a few minutes to stir up any heavier protozoa and food particles that had sedimented. The gas stream was maintained during the sampling to prevent the entrance of air and the gas flow adjusted to its normal rate once the bung had been replaced.

Analyses. Total volatile fatty acids were estimated by the method of Annison (1954*a*). Non-protein nitrogen was determined by the method of Lang (1958).

For the extraction of long-chain fatty acids, samples (4 ml.) were removed from the artificial rumen and mixed thoroughly with 200 ml. of chloroform–methanol (2:1, v/v). After being centrifuged, the extract was evaporated to dryness and the residue hydrolysed for 60 min. at 95–100° with N-NaOH (20 ml.). The non-saponifiable material was removed by extraction with light petroleum (b.p. 60–80°); the saponifiable material was acidified with 5N-HCl and the free fatty acids were extracted with light petroleum which was then washed twice with water. The light petroleum was shaken three times with 4% (w/v) K_2CO_3 (50 ml.), and the alkaline extract was acidified with 5N-HCl and the fatty acids were extracted into light petroleum, which was washed twice with water (50 ml.). The organic phase was evaporated to dryness and the residue containing the long-chain fatty acids dissolved in ether and dried with anhydrous Na_2SO_4.

The fatty acids were analysed by using a Pye Argon gas chromatograph. The methyl esters were examined on Apiezon L and poly(ethylene glycol adipate) columns and identified by comparison of their retention times with those of known markers under identical conditions. The percentage composition of the fatty acid mixture was determined by measuring the areas of peaks with a planimeter.

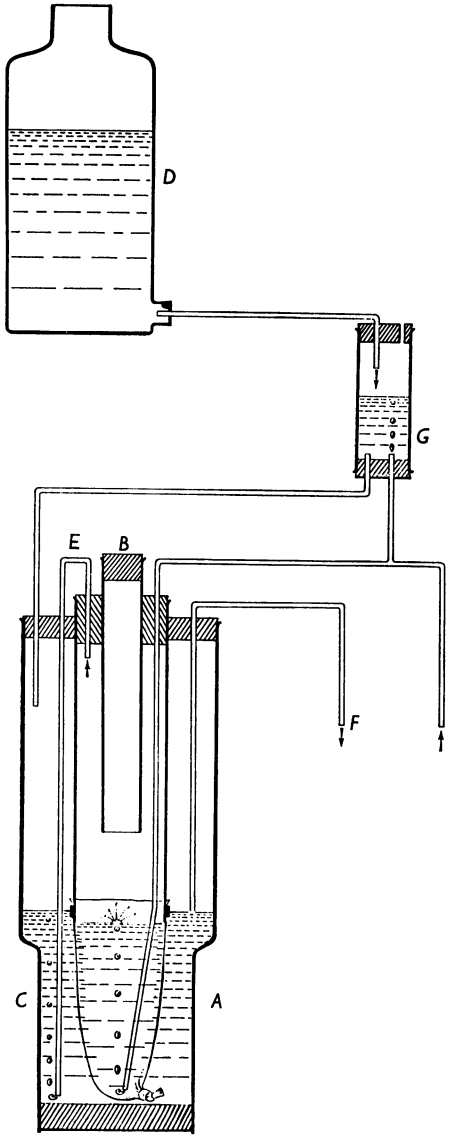

Fig. 1. Artificial rumen apparatus. Details are given in the text.

RESULTS AND DISCUSSION

Validity of the apparatus as a functioning rumen. When the contents of the artificial rumen were examined under a microscope after an experimental run, there was no visible difference between the appearance and motility of the micro-organisms and those of a preparation obtained freshly from the rumen of the same sheep. However, in the present state of knowledge of rumen bacteriology and protozoology, it is almost impossible to examine the contents of an artificial rumen and state that the types and numbers of viable micro-organisms are the same as those in the living animal (see, however, Davey *et al.* 1960). Moreover, there is almost certainly a variation in the numbers of such micro-organisms in the intact animal depending on the type of diet and the time of feeding (Bryant & Robinson, 1961).

Consequently the tests of validity performed were largely biochemical, i.e. to ascertain whether the micro-organisms *in vitro* could digest and change the foodstuffs in a similar manner to those in the intact rumen *in vivo*. Three of the most important digestive reactions occurring in the rumen are the degradation of cellulose with the production of short-chain fatty acids, the hydrolysis of proteins to give amino acids and eventually free ammonia, and the hydrogenation of unsaturated fatty acids to produce saturated fatty acids which are eventually deposited in the depot fats. Each of these processes has been examined in the artificial rumen.

Production of volatile fatty acids. Fig. 2 shows the formation of volatile fatty acids when the artificial rumen was given 1 g. of hay diet (equivalent to 100 g. in the intact sheep). The fractions collected were analysed for their volatile fatty acid content and the concentration in the perfusion fluid was subtracted in order to calculate the contribution from the digestion performed by the micro-organisms. In Fig. 2 (*a*) the production of short-chain fatty acid in the system is plotted against time. Before the feeding a very slow formation of volatile fatty acid is apparent, presumably coming from the metabolism of residual foodstuffs; feeding

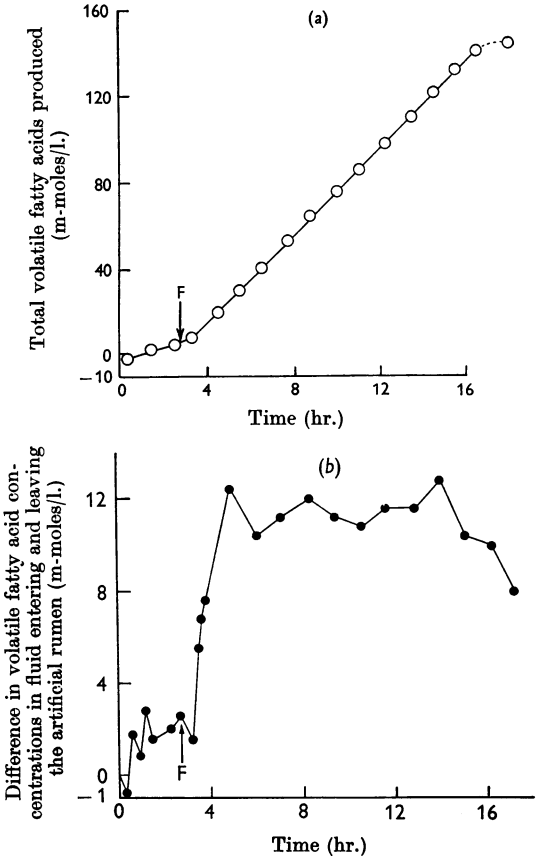

Fig. 2. (*a*) Total volatile fatty acid production by rumen micro-organisms in the artificial rumen after giving 1 g. of the diet at F. (*b*) Rate of volatile fatty acid production in the artificial rumen after giving 1 g. of the diet at F. Details are given in the text.

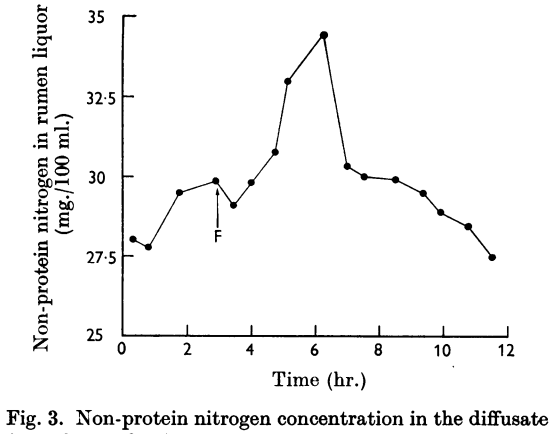

Fig. 3. Non-protein nitrogen concentration in the diffusate from the artificial rumen. The micro-organisms were given 1 g. of the diet at F. Details are given in the text.

produces an almost immediate increase in the production which is maintained at a constant rate until the substrate becomes exhausted once more (Fig. 2*b*). At this point calculation shows that the amount of fatty acid formed is in good agreement with the theoretical value expected. The constant rate of production suggests that the system is maintaining its capacity to degrade cellulose throughout the experimental period.

In the intact sheep the volatile fatty acids produced in the rumen are rapidly absorbed into the blood stream and are quickly metabolized by the tissues. Nevertheless, the rapid increase in concentration of volatile fatty acids in the perfusion fluid to a maximum at 2 hr. after the feeding compares well with the results reported by Annison (1954*b*), for the intact sheep. Here the maximum blood concentrations after feeding hay were reached within a few hours.

Formation of non-protein nitrogen. The release of non-protein nitrogen from the hay feed is illustrated in Fig. 3. Although the increased nitrogen concentration of the perfusion fluid is superimposed on a high basic concentration, it can be seen that the release of soluble nitrogen is almost complete 3 hr. after the feeding. McDonald (1952) reported maximal concentrations of non-protein nitrogen in the rumen contents about $2\frac{1}{2}$ hr. after feeding meadow hay, and a similar rapid increase of non-protein nitrogen in ox rumen after feeding hay may be calculated from the results given by Mangan, Johns & Bailey (1959).

Hydrogenation of unsaturated fatty acids. The fatty acids of pasture grass contain a high percentage of linolenic acid (Garton, 1960). The intact rumen contains hydrogenases that hydrogenate this fatty acid to form stearic acid (Garton, 1961). Fig. 4 shows the percentage C_{18}-fatty acid composition of the contents of an artificial rumen at various times after feeding 1 g. of hay. A control artificial rumen was also examined which was not fed during the experimental period.

In rumen contents obtained from a sheep deprived of food overnight the predominant fatty acid is stearic acid. The ingestion of hay with its high content of C_{18} unsaturated fatty acids (mainly linolenic acid but with some linoleic acid and oleic acid) caused a big decrease in the percentage of stearic acid in the fatty acids of the artificial rumen contents. During the experimental period the percentage of C_{18} unsaturated fatty acids progressively decreased and there was a concomitant increase in the percentage of stearic acid. This indicated that a slow hydrogenation was occurring and causing the conversion of C_{18} unsaturated fatty acids into stearic acid.

Experiments with the intact sheep in which an equivalent feed of hay was given indicated a very

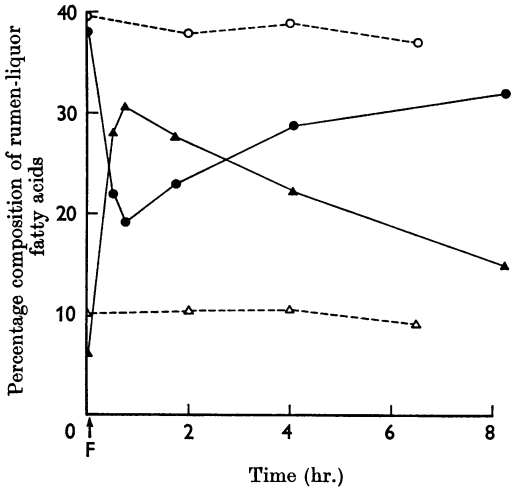

Fig. 4. Comparison of C_{18} fatty acids in the artificial rumen with and without feeding. The artificial rumen containing 55 ml. of strained rumen contents was given 1 g. of the diet at F: ▲, unsaturated C_{18} acids; ●, stearic acid. A control experiment (no feeding) was also carried out: △, unsaturated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

T.W.S. is indebted to the International Atomic Energy Agency for a grant for maintenance and isotopic equipment.

REFERENCES

Annison, E. F. (1954*a*). *Biochem. J.* **57**, 400.
Annison, E. F. (1954*b*). *Biochem. J.* **58**, 670.
Bryant, M. P. (1959). *Bact. Rev.* **23**, 125.
Bryant, M. P. & Robinson, I. M. (1961). *J. Dairy Sci.* **44**, 1446.
Davey, L. A., Cheeseman, G. C. & Briggs, C. A. E. (1960). *J. agric. Sci.* **55**, 155.
Elsden, S. R. (1952). *Proc. Roy. Soc.* B, **139**, 193.
Garton, G. A. (1960). *Nature, Lond.*, **187**, 511.
Garton, G. A. (1961). In *Digestive Physiology and Nutrition of the Ruminant*, p. 140. Ed. by D. Lewis. London: Butterworths.
Lang, C. A. (1958). *Analyt. Chem.* **30**, 1692.
Louw, J. G., Williams, H. H. & Maynard, L. A. (1949). *Science*, **110**, 478.
McDonald, I. W. (1952). *Biochem. J.* **51**, 86.
Mangan, J. L., Johns, A. T. & Bailey, R. W. (1959). *N.Z. J. agric. Res.* **2**, 342.
Warner, A. C. I. (1956). *J. gen. Microbiol.* **14**, 733.

Biochem. J. (1964) **90**, 12

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

BY T. W. SCOTT, P. F. V. WARD AND R. M. C. DAWSON
Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 24 *June* 1963)

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-