Criteria for Establishing the Validity of *in vitro* Studies with Rumen Micro-organisms in so-called Artificial Rumen Systems

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SUMMARY: Several criteria of normal rumen function which can be applied to *in vitro* studies with the whole rumen microbial population are suggested. These include: the maintenance of numbers and normal appearance of the bacteria, selenomonads and protozoa of the rumen; the maintenance of normal rates of digestion of cellulose, starch and protein, and of normal interactions between these; the ability to predict quantitative results *in vivo*. An 'artificial rumen' was constructed, consisting of a cellophan sac containing rumen liquor and substrate dialysing against a complex mineral solution whose composition was based on that found in rumen liquor, the whole being incubated at 39° in an atmosphere of nitrogen and carbon dioxide. This system was shown to meet the criteria which are suggested, with reasonable success for periods of about 8 hr.; over longer periods an increasing failure to meet the biological criteria was seen. For the microbial population to remain normal in numbers and activity it was shown to be necessary to use as test substrate *in vitro* only substances similar to the diet fed to the animal from which the rumen liquor inoculum was taken.

Of recent years there has been an increasing interest in the biochemistry and microbiology of the rumen. Because of the complexity of the rumen environment, many *in vitro* techniques have been applied including the use of: pure cultures of bacteria isolated from the rumen; washed suspensions of the whole or part of the rumen microbial population; cell-free enzyme preparations; whole rumen liquor under conditions which permit or encourage microbial multiplication. Under these last conditions, sometimes known as the 'artificial rumen' technique, it is essential to ensure that the microbial multiplication which takes place should proceed in a fashion similar to that found in the rumen of the living animal. There is normally present in the rumen a very large number of microbial species of widely differing physiological needs and capabilities, some found apparently only in the rumen and some found widespread in foodstuffs, soil, etc.; some of these microbial species may be expected to be able to multiply in almost any environment, and to metabolize almost any substrate that is likely to be tested. When, however, the environment or substrate is not that found in the rumen of the animal from which the rumen liquor inoculum was taken, then, in time, the results of this multiplication and metabolism may bear little or no relation to events in the rumen *in vivo*, since the organisms which multiply may be simply those most suited to the experimental conditions, and may well be only chance contaminants originally present in the rumen

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in quite small numbers. Since incubation periods of several days have frequently been used, the danger of this happening is very real.

Four main types of artificial rumen systems have been described: (1) undiluted or only slightly diluted rumen liquor is incubated with substrate in an all-glass impermeable system as used principally by Pearson & Smith (1943), Quin (1943) or Gray, Pilgrim & Weller (1951) and their respective co-workers; (2) whole rumen liquor is diluted to about half strength with a mineral solution resembling ruminant saliva in composition, and incubated with substrate in an impermeable system, as first described by Burroughs, Frank, Gerlaugh & Bethke (1950) but since used, with variations in detail, by many other workers; (3) various fractions of rumen liquor are used in an impermeable system, such as rumen liquor freed from protozoa by centrifuging (McNaught, 1951) or a suspension of all the rumen micro-organisms in a mineral solution (Marston, 1948); (4) rumen liquor, usually whole and undiluted, with substrate in a semi-permeable container, is dialysed against a mineral solution, as described by Louw, Williams & Maynard (1949), Huhtanen & Gall (1952) and Wasserman, Duncan, Churchill & Huffman (1952). Despite extensive use of some of these systems, not much has been done to establish the validity of the results in terms of normal rumen function, and some of what has been done is open to criticism.

Pearson & Smith (1943), on the basis of findings by the late F. Baker, who used formolized samples, claimed that their system remained normal for 2-4 hr. and that numbers and types of, mainly, the iodophilic bacteria showed no significant changes during that time; with the same technique, McNaught & Owen (1949) showed that that concentration of *o*-phenanthroline which suppressed urea utilization *in vitro* corresponded to a concentration of ferrous ion of the same order as that found *in vivo*. With a similar system with rumen liquor freed from protozoa McNaught (1951) found that the CO_2/CH_4 ratio in the evolved gases had an average value similar to that found *in vivo*, though the range of values was larger. Quin (1943) and McAnally (1943) showed that the rate of gas output following addition of glucose was similar *in vivo* and *in vitro*, but the incubation periods observed were very short.

Gray *et al.* (1951) successfully applied a number of criteria of normal rumen function to their system: the microscopically observed activity of the protozoa; the ratio of methane produced to fodder supplied; the digestibility of cellulose and pentosans; these were all similar *in vitro* and *in vivo*, but the authors noted that the rates of digestion of these substrates and of methane production were all only half those found *in vivo*. It is perhaps unfortunate that they used as buffer ammonium carbonate equivalent to 103 mg. N/100 ml., a concentration of ammonia very rarely found *in vivo* in the rumens of healthy animals even when fed a diet rich in a readily attacked protein (Gray & Pilgrim, 1952).

Burroughs, Frank, Gerlaugh & Bethke (1950) and Burroughs, Headley, Bethke & Gerlaugh (1950) in their system with successive fermentation periods each started by a 50 % (v/v) inoculum of material from the preceding fermentation, claimed that there were no marked changes in numbers, size or predominant types of bacteria throughout, but that some of the types of

protozoa failed to survive. However, the main criterion of normal rumen function relied on by these workers was the high degree of cellulose digestion obtained; but two points must be noted. First, the absolute amount of cellulose used was small, about 3 g./l. artificial rumen contents in early experiments, later 10 g./l., or occasionally up to 20 g./l., though animals fed high roughage diets, such as were used to supply the inoculum for these experiments, would receive perhaps 50 g. cellulose/l. rumen contents/day, as well as hemicelluloses, etc., which might well involve the same digestive enzymes, so that the actual amount, in g. cellulose/l., digested/day in the artificial rumen was much less than was digested *in vivo*. Secondly, the criterion relied on, namely the digestibility of cellulose over several fermentation periods, does not seem very reproducible. In two successive papers where detailed figures are given (Arias, Burroughs, Gerlaugh & Bethke, 1951; Burroughs, Latona, De Paul, Gerlaugh & Bethke, 1951), when examination is made of the results from the control flasks containing 9 g. cellulose/900 ml. diluted rumen liquor only, and followed through four successive fermentations (there are five such in the first paper and six in the second), a statistically significant increase in cellulose digestibility can be seen during the second fermentation period, and a significant fall in the third period, while in the fourth period there was a highly significant but unexplained difference between the results reported in the first paper (where there was a marked drop in digestibility), and those in the second paper (where there was little if any decrease).

Using a somewhat similar system, though with only one fermentation period of about 40 hr., Brooks, Garner, Gehrke, Muhrer & Pfander (1954) claimed that the numbers of bacteria before and after incubation were approximately equal, and that the effects of added fat on cellulose digestion *in vivo* and *in vitro* were very similar. However, examination of the figures shows that a dose of 32 g. corn oil/sheep decreased cellulose digestibility from 41.9 to 20.0 %, while a dose of 160 mg. corn oil/25 ml. diluted rumen liquor *in vitro* (the same concentration, assuming a rumen volume of 5000 ml.) decreased cellulose digestibility from 36.2 to 2.2 %, a much more marked effect. Brooks, Garner, Muhrer & Pfander (1954) also tested the effects of various steroids on cellulose digestibility *in vivo* and *in vitro*, and again a more marked effect was obtained *in vitro*.

Louw *et al.* (1949) showed that the semipermeable artificial rumen permitted better digestion of cellulose than an impermeable system with the same inoculum. Gall & Glaws (1951) and Huhtanen, Saunders & Gall (1954) showed that the bacteria seen in a Gram-stained film or grown in anaerobic culture were more nearly similar at the end and the beginning of incubation, and the protozoa were more motile throughout, with a semipermeable system as compared with an impermeable system.

In summary, then, both biological and chemical criteria have been used in attempts to establish the validity of the results obtained with these artificial rumen systems. Biologically, attention has been paid to: motility of the micro-organisms, particularly the protozoa; predominant morphological or cultural types of bacteria; in a few cases, numbers of bacteria, though no detailed

figures have been published. Chemically, most attention has been paid to the proportion of cellulose digested, though other relative values have been used; little attention has been given to the actual rates of digestion of substrate. It should be noted that in no case where incubation has been continued for more than a very few hours has complete success been reached in meeting even the few criteria that most authors have been content to adopt. It is the intention of this paper to discuss a number of criteria of normal rumen function and to apply these to an artificial rumen system based on that of Louw *et al.* (1949).

METHODS

Animals. Cheviot ewes or wethers were fitted with rumen fistulas by Dr A. T. Phillipson. Except where otherwise mentioned, the diet was: 300 g. hay, 300 g. groundnut meal and 300 g. flaked maize/day, fed in two portions at 07.30 and 19.30 hr. Samples of rumen liquor were removed at 07.00 hr. strained through eight thicknesses of surgical gauze and used without delay.

Materials. Groundnut meal, herring meal, Paisley meal and casein were as described by Annison, Chalmers, Marshall & Synge (1954). Starch used in *in vitro* tests was Soluble Starch (British Drug Houses, Ltd.); potato starch was used in feeding experiments. Cellulose was filter-paper ground in a hammer mill. All meals, etc., used in *in vitro* work were finely powdered in a hammer mill before use.

Chemical estimations. Ammonia-N was estimated by adding 1.0 ml. rumen liquor or other fluid to 1.0 ml. 0.1 N-HCl, diluting to 5.0 ml. and filtering (McDonald, 1952), liberating the NH_3 in the filtrate with K_2CO_3 according to Conway & O'Malley (1942) and absorbing it in the boric acid indicator solution diluted with water to half strength (E. F. Annison & J. C. Wood, private communication). This diluted indicator solution gives a much more sensitive end-point for amounts of ammonia up to about 0.05 mg. NH_3 -N, and will quantitatively absorb up to about 0.16 mg. NH_3 -N. Volatile fatty acid was estimated by steam distillation of 2.0 ml. of the HCl filtrate obtained above with 1.0 ml. syrupy H_3PO_4 in the apparatus of Markham (1942); 50–60 ml. of distillate were collected, aerated with CO_2 -free air for at least 3 min. and titrated with 0.01 N-NaOH (CO_2 -free). An approximate estimate of starch in rumen liquor was made according to a method of Dr P. N. Hobson (private communication). Rumen liquor (2.0 ml.) was placed with 0.6 ml. 5 N-NaOH in a 10 ml. measuring flask, heated in a boiling water-bath for 20 min., cooled and diluted to the mark with water. After standing for a few minutes, the mixture was centrifuged for 20 min. at 2500 r.p.m. and 1.0 ml. of the supernatant fluid added to c. 60 ml. water; 4 drops 5 N- H_2SO_4 and 1.0 ml. Spekker iodine (0.2 % I_2 in 2.0 % KI, w/v) were added, the whole made up to 100 ml. and the colour measured immediately in an EEL portable photoelectric colorimeter, using the red filter. Experiment showed that each scale division (0.01 optical density unit) was approximately equivalent to 0.1 g. starch/100 ml. rumen liquor.

Microbial counts. Strained rumen liquor was diluted 1/5 with 10 % (v/v)

formalin (Baker, 1943), and after shaking, the protozoa were counted in a Manners counting chamber (0.2 mm. deep) and the bacteria in a Helber counting chamber (0.02 mm. deep) (counting chambers supplied by Hawksley & Sons Ltd., London). Phase-contrast microscopy was used throughout. By counting about 2000 organisms of each of the numerically more important types of micro-organism, replicate counts were made to agree within $\pm 5\%$. Special precautions were needed with the Helber counting chamber, since its depth was less than at least two dimensions of the larger protozoa; the actual depth of fluid in the chamber was tested each time by means of the calibrated fine-focusing mechanism of the microscope. Any preparation which showed a burst holotrich protozoon on low-power examination was rejected, as the grains of protozoal polysaccharide and other intracellular particles liberated from such a burst organism were at times difficult to distinguish microscopically from bacteria.

Artificial rumen apparatus. The apparatus (Fig. 1) consisted of a piece of glass tubing, 20×5 cm. closed with rubber bungs at each end and containing a cellophan dialysing sac, 12×3 cm., also closed with rubber bungs. This sac contained usually 50 ml. rumen liquor and the substrate, previously moistened with the mineral solution described below. The outer chamber was filled with the mineral dialysing solution to the same level as the rumen liquor, the volume being noted. A slow stream of nitrogen containing 5% carbon dioxide (British Oxygen Co. Ltd.) was bubbled through both solutions, partly to avoid loss of material through frothing, which was troublesome with some substrates, any material carried over in froth being measured in the ordinary analysis of the mineral solution. The pH values of the solutions in both compartments were adjusted to between 6 and 7 when necessary, using $m\text{-H}_3\text{PO}_4$ or a mixture of $0.5\text{ M-K}_2\text{HPO}_4 + 0.5\text{ M-Na}_2\text{CO}_3$; when the substrate approximated the composition of a normal diet, little adjustment was needed, but when single substrates, particularly starch, were used, considerable alterations of pH value occurred. The whole apparatus was immersed in a water-bath at 39° .

Fig. 1. Artificial rumen apparatus (about $\frac{1}{2}$ scale). 1, Inlet for gas mixture; 2, access to mineral solution and outlet for gases; 3, access to inner sac; 4, duct for gases; 5, cellophan dialysing membrane; 6, rumen liquor and substrate; 7, complex mineral solution and products of diffusion from rumen liquor; 8, glass beads to facilitate mixing by rotating the whole apparatus by hand; 9, outer glass container.

At the conclusion of the experiment, the volumes of fluid in both compartments were measured, and the volumes at intermediate times were calculated, allowing for known volumes removed as samples and assuming that any flow due to osmotic differences, etc., was constant. The ratios of the outer to the inner volumes were then calculated; in the apparatus described, these ratios were always between 2.9 and 4.1, usually about 3.5. The total 'concentration' of any metabolite was then calculated as the concentration inside the dialysing sac + the product of the concentration in the outside compartment (less the value at zero time) and the above ratio; this gives in effect what would have been the concentration of the metabolite in the dialysing sac if none had diffused away. All concentrations given in this paper for diffusible substances are calculated in this way, so that, for example, the concentrations of ammonia actually in contact with the micro-organisms would be considerably less than the figures given, perhaps one-third, and consequently well within physiological limits at all times.

The influence of the mineral base used in work with rumen micro-organisms *in vitro* has been inadequately examined, it being customary to use either a simple buffer or a complex solution of composition similar to saliva. In the present work, a similar solution (solution I of Table 1), was used for all except

Table 1. *Composition of mineral dialysing solutions, compared with the composition of saliva and rumen liquor of sheep*

Element or ion	Concentration (mg./100 ml.)			
	Solution I	Solution II	Saliva*	Rumen liquor†
K	74	117	12-46	99-175
Na	242	197	352-462	137-202
NH ₄ -N	14	14	9-35	—
Ca	4	10	0.2-3.0	10-21
Mg	2.4	6	0.4-1.1	7-20
PO ₄ -P	155	101	19-129	28-81
SO ₄ -S	3.2	24	—	—
Cl	126	87	19-238	35-92
HCO ₃ ⁻	Saturated	Saturated	Saturated	Saturated
Fe	0.2	0.2	—	—
Mn	0.13	0.13	—	—
Zn	0.1	0.1	—	—
Co	0.05	0.05	—	—
Cu	0.05	0.05	—	—
Acetate ⁻	180	216	—	—

Solutions I and II also contained 2 ml./l. of the 10% cysteine solution of Huhtanen, Rogers & Gall (1950).

* See McDougall (1948).

† See Phillipson (1953).

the last two experiments described. On noting the differences between the composition of this mixture and that of rumen liquor (Phillipson, 1953), the mixture was amended accordingly, and the following differences in functioning of artificial rumens dialysing against these two solutions (solutions I and II of

Table 1) were noted: motility of the protozoa and the selenomonads* was better maintained with solution II; the rate of ammonia production in the absence of added substrate was slightly but significantly lower; the rate of ammonia production in the presence of added casein was slightly but significantly higher with solution II than with solution I. Solution II was therefore used in the remaining experiments reported here. Similar differences were noted when comparing solution II with a simple phosphate NaCl buffer. After completion of this work, the influence of various mixtures of volatile fatty acids on one of the more important rumen bacterial species was reported by Bryant & Doetsch (1954), and some slight improvements appeared to be made on replacing some of the chloride and acetate in solution II with propionate, isobutyrate, valerate and isovalerate in the artificial rumen system at concentrations within the limits described by Annison (1954). In assessing the importance of some of these substances in the dialysing system, it must be realized that, owing to the closed nature of the system, no substance could diffuse out of the dialysing sac to give a final concentration lower than about a quarter of its initial value.

CRITERIA OF VALIDITY OF THE ARTIFICIAL RUMEN USED

Numbers of micro-organisms

The numbers of micro-organisms before and after incubation cannot be used to estimate true mean generation times owing to the complexity of the system: it is probable, though not proven, that the protozoa consume bacteria in large numbers either to satisfy nutritional needs or simply as attached to food particles; protozoa can be seen to consume other protozoa; bacteria and possibly selenomonads can be seen to consume protozoa; the quantitative significance of these processes in the economy of the rumen is, however, quite unknown. Table 2 shows that in the presence, though not in the absence, of substrate the numbers of bacteria and protozoa were maintained approximately constant in the artificial rumen for 7 hr. The decrease in the number of selenomonads appears to be due to the nature of the substrate used since it was noted in several experiments with casein + starch as substrate, whereas in an experiment using starch + cellulose + purified groundnut meal protein there was a slight rise in numbers over 8 hr. Too much significance is not claimed for counts of this nature, except when using as substrate the diet of the animal from whose rumen the inoculum was taken and comparing changes in microbial numbers *in vivo* and *in vitro*; they do, however, seem to show that no noticeable change occurred in the balance of micro-organisms within the time stated.

Motility of micro-organisms

In rumen liquor examined immediately after removal from the animal, about 90 % of the protozoa and 80 % of the selenomonads appeared actively motile. After 8 hr. in the artificial rumen, the motility of the holotrich protozoa appeared unaffected; about 60 % of the entodinia were actively motile, about

* Term used here to include organisms 3, 4 and 5 of Moir & Masson (1952); see also Lessel & Breed (1954); Judicial Commission (1955).

25 % sluggishly motile (in the rumen contents of the animals used in this work other genera of oligotrich ciliate protozoa were either absent or present in too low numbers to be accurately counted). About 40–50 % of the selenomonads were actively motile. Owing to the difficulty of identifying species with certainty, motility in bacteria was not examined. The motility of the protozoa and selenomonads did not appear to depend to any great extent on the presence

Table 2. *Numbers of micro-organisms present in rumen liquor before and after 7 hr. incubation in the artificial rumen apparatus*

Organism	Counts (no./ml.)			
	No added substrate		1 % casein and 1.8 % starch added	
	Initial	Final	Initial	Final
Total protozoa $\times 10^{-6}$	1.92	1.64	1.78	1.84
<i>Entodinium nanellum</i> * group $\times 10^{-5}$	7.4	6.9	7.7	6.7
<i>Entodinium longinucleatum</i> * group $\times 10^{-5}$	2.9	2.8	3.2	3.3
<i>Entodinium caudatum</i> * group $\times 10^{-5}$	5.0	4.1	4.6	4.9
Total selenomonads $\times 10^{-8}$	3.9	3.0	3.2	2.4
Total bacteria $\times 10^{-10}$	3.5	2.7	3.4	3.6
Chains of large streptococci† $\times 10^{-7}$	1.9	1.5	2.0	2.4

* Species differentiation by phase-contrast examination alone is almost certainly unsatisfactory, but many of the protozoa seen can be placed in groups resembling species as described by Bhatia (1936). Other, unidentifiable, *Entodinia* spp. were also seen, and the total number of entodinias constituted about 98 % of the total protozoal count in this instance.

† Organism 27 of Moir & Masson (1952).

or absence of substrate, at least within the period studied, possibly owing to the removal of dead organisms by living ones. Motility appears to be a sensitive criterion of normal rumen function, but care must be exercised to ensure maintenance of temperature and anaerobiosis of the specimen during examination.

Proportion of dividing protozoa

It was thought that the proportion of organisms showing signs of being about to divide might be a sensitive measure of the viability of the protozoa. Unfortunately, this proportion fluctuates *in vivo* for causes so far not determined. In specimens of rumen liquor, taken at various times from one sheep on one diet, the proportion of entodinias dividing was between 1.2 and 1.9 % on seven occasions, between 0.6 and 1.2 % on five occasions and just over 3 % once; these fluctuations did not appear to depend to any great extent on the time after feeding at which the sample was taken. Nevertheless, it is probably significant that in all experiments where this proportion has been estimated, it has decreased to about half its initial value after 8 hr. in the artificial rumen. The dividing organisms present at the end of the experiment could not have been those seen at the beginning: if dividing entodinias were followed about a slide kept at about 39° under the microscope, division was seen to be completed in 15–25 min. It may be noted that a median value of 1.5 % organisms

dividing and taking 20 min. to complete division indicates a mean generation time of 22 hr., about the value found by Hungate (1942) from his work with cultures.

Effect of long incubation periods

Over a period of nearly 4 days, on some five occasions each day, about 15–20 % of the contents of the dialysing sac were removed and replaced with mineral solution; 3 times/day the contents of the outer compartment were completely renewed; twice a day a mixture equivalent to the animal's feed was added to the sac; except for a period of some 11 hr. each night when no attention was given, the system was mixed and neutralized at intervals. The experiment was terminated when the cellophan dialysing sac split along the line of folding. Throughout the experiment, the protozoa, selenomonads and spirilla were actively motile, except in the first specimens examined in the morning, when motility was sluggish. All morphological types of micro-organism present at the start of the experiment were present at the finish, though the proportion of spirilla and of the large streptococci increased; the latter organism has not yet been cultivated outside the rumen, suggesting that it has some special nutritional needs supplied presumably by commensal growth of other micro-organisms, so that an increase in numbers in this experiment would not suggest a gross abnormality in the medium. The mechanical dilution was such that the final concentration was 0.066 of the initial; the final number of entodinas was 0.24 of the initial, so that there was a net multiplication of 3.6 times. That is, despite the long period during the night when no attention was given to the apparatus, the microbial population was still reasonably typical of the rumen over 4 days. Long-term experiments not involving progressive dilution, etc., that is where rumen liquor was incubated with substrate for 24–48 hr., without any other additions or removals, showed a much more rapid deterioration of the microbial population, the protozoa and several morphologically distinctive bacterial species, particularly *Oscillospira guilliermondii* or organism 1, and organism 25 of Moir & Masson (1952), markedly decreasing in numbers within 24 hr.

Cellulose digestion

Although cellulose digestion is one of the most frequently used criteria of normal functioning of artificial rumen systems, there are certain disadvantages in laying stress on this, even though it is one of the most characteristic functions of the rumen. There is, first, considerable doubt as to the actual rate of cellulolysis in the rumen as distinct from an overall rate for the whole animal. The rate-limiting step in cellulose digestion is unknown; if it should occur at a very early stage, where the products would still be measured as cellulose by most techniques in common use, it might well be that the rate of digestion of the cellulosic contents of the rumen as ordinarily measured (and roughage on the average probably remains in the rumen 24 hr. or more), might be very different from the rate of digestion of cellulose newly introduced into an artificial system. Moreover, since it is practically essential to strain the rumen

liquor for use *in vitro*, some diminution of the rate of digestion of cellulose is to be expected, since many of the cellulolytic micro-organisms would remain attached to the large plant particles removed in straining. The sheep used to provide the inoculum in the present experiments was fed a high concentrate diet, containing probably less than 200 g. cellulose/day; since the diet was of a type found by Head (1953) to depress cellulose digestibility, probably considerably less than 100 g. cellulose was being digested each day. Addition of 2.0 g. cellulose to 50 ml. rumen liquor in the artificial rumen apparatus resulted in the production of 1.1 mequiv., probably 80 or more mg. volatile fatty acid in 8 hr.; 1 g. volatile fatty acid is produced from about 2 g. cellulose (Carroll & Hungate, 1954), so that the rate of cellulose digestion in this apparatus was about 10 g./l. rumen liquor/day, or about 50 g./day for the animal. Considering the various assumptions made, this agreement seems fairly good. This *in vitro* rate of cellulose digestion is exceeded only by the figure of 18 g./l./day given by Louw *et al.* (1949).

Starch digestion

The rate of starch digestion *in vitro* as measured by the rate of disappearance of starch appears to depend considerably on the diet of the animal from which the rumen liquor inoculum was taken, as shown in Table 3. Dr P. N. Hobson (private communication) found in the case of sheep no. 1054 that few starch grains were visible microscopically in the rumen contents 12 hr. after feeding 100 g. potato starch, a rate of starch digestion probably equivalent to the 2.0 g./l. rumen contents/hr. found *in vitro*.

Table 3. *Effect of diet of animal on rate of starch digestion in vitro*

Animal	Daily diet	Rate of starch digestion, g./l. rumen contents/hr.
969	900 g. hay	0.5
43	300 g. hay, 300 g. flaked maize, 300 g. groundnut meal	1.0
377	600 g. hay, 300 g. concentrates	1.5
70	600 g. hay, 300 g. flaked maize	1.0
416	600 g. hay, 300 g. maize gluten	1.3
1054	300 g. hay, 200 g. potato starch, 500 g. concentrates	2.0

Casein digestion

Dr I. W. McDonald (private communication) found that when 100 g. casein was fed to a sheep, the last traces of bound phosphate, presumed to come from the casein, passed through the abomasum about 8–10 hr. after feeding. The peak in the curves for ammonia production in the rumen found by Chalmers and colleagues (Annison *et al.* 1954; Chalmers & Synge, 1954; Chalmers, Cuthbertson & Synge, 1954) usually occurred about 4–5 hr. after feeding 50–100 g. casein. It seems reasonable to suppose that the time for complete digestion in the rumen is intermediate between these values, say 6–8 hr. In the artificial rumen, the curve for ammonia production from added casein first rises at a rate independent of the amount added, and then, at a time which depends on

that amount, flattens out to a rate of ammonia production equal to that found in the absence of added substrate (see Fig. 2). It seems probable that this point of inflexion corresponds to the completion of digestion of the casein. In a considerable number of experiments ammonia production due to 0.7 g. casein in 50 ml. rumen liquor ceased after 5-8 hr., corresponding to an amount of 70 g. casein/sheep digested in a similar time, or a rate of casein digestion of about 0.3 g. N/l./hr.

Fig. 2

Fig. 2. Ammonia production from varying amounts of casein in the artificial rumen apparatus. ●, no added substrate; ○, 0.3 g. casein/artificial rumen (70 mg. N/100 ml.); +, 0.7 g. casein/artificial rumen (180 mg. N/100 ml.); ×, 1.2 g. casein/artificial rumen (320 mg. N/100 ml.).

Fig. 3

Fig. 3. Rates of production of ammonia from various protein foodstuffs in the artificial rumen. ●, no added substrate; ■, 2.2 g. paisley meal/artificial rumen (184 mg. N/100 ml.); ○, 0.8 g. herring meal/artificial rumen (178 mg. N/100 ml.); +, 1.1 g. groundnut meal/artificial rumen (180 mg. N/100 ml.); ×, 0.7 g. casein/artificial rumen (184 mg. N/100 ml.).

Ammonia production from other proteins

The relative amounts of ammonia produced in the artificial rumen from groundnut meal, herring meal, Paisley meal and casein (Fig. 3) appear to correspond to the relative amounts found to be produced *in vivo* by Chalmers and colleagues, namely, slightly less from groundnut meal than from casein, considerably less from herring meal, and little, if any, from Paisley meal.

Interaction between starch and casein

It has been known for some time that the addition of starch lowers the ammonia concentration in the rumen, whether the ammonia is largely produced from urea (Mills, Booth, Bohstedt & Hart, 1942) or from protein (McDonald, 1952; Annison *et al.* 1954). This was also found in the artificial rumen (Table 4), where the addition of starch lowered endogenous ammonia production nearly as much as total ammonia production from casein.

The end products of digestion

The nature of relative quantities of the end products of digestion have been used by various workers to demonstrate normality of function of an *in vitro* system. In the present work, it was found that ammonia and volatile fatty acids were produced in roughly equimolar quantities (Table 4). In other experiments, the output of ammonia-N, in m-equiv./100 ml., from

Table 4. *Digestion of starch and casein, separately or together, in the artificial rumen, in 9 hr.*

	Contents of artificial rumen apparatus: 50 ml. rumen liquor with			
	No added substrate	1.8 % (w/v) starch	1.0 % (w/v) casein	1.8 % (w/v) starch and 1.0 % (w/v) casein
Starch (g./100 ml.)				
Initial	0.20	1.83	0.20	2.00
Final	0.00	0.70	0.03	0.60
Amount digested -- blank	—	0.93	—	1.23
Volatile fatty acid (m-equiv./100 ml.)				
Initial	5.9	6.0	6.0	6.1
Final	16.5	25.0	24.7	32.8
Amount produced -- blank	—	8.4	8.1	16.1
Ammonia-N (mg./100 ml.)				
Initial	22.8	22.8	22.8	22.8
Final	57.8	37.5	140.3	116.2
Amount produced -- blank	—	-20.3	+82.5	+58.4

12.5 m-equiv. N casein/100 ml., was 5.9, 6.4, 5.8, 5.7 and 5.9; the output of volatile fatty acid in m-equiv./100 ml. in the same experiments was respectively 8.1, 5.3, 7.9, 6.7 and 5.0. These figures correspond to the roughly equimolar amounts of ammonia and volatile fatty acids found in the rumen after feeding several diets by el-Shazly (1952) and Annison (1954). It was also found that considerable amounts of volatile fatty acid were produced from starch, concentrates, hay and cellulose in the artificial rumen.

Quantitative prediction of in vivo results

Two rations, E and H, were composed such that they gave equal ammonia production in the artificial rumen, when rumen liquor from sheep 70 fed the standard groundnut meal diet was used as inoculum. The composition of these rations is given in Table 5; they were fed to two sheep, 377 and 416, in turn. Three weeks after beginning the diet, the rumen ammonia production *in vivo* was determined, following the methods of Chalmers *et al.* (1954). The results (Figs. 4, 5) showed that reasonable prediction had been obtained, allowing for some variation between the two animals. At the same time, a third diet, G, was tested; this also had given similar ammonia production in the preliminary *in vitro* test, but *in vivo*, markedly less ammonia was produced from diet G than from either of diets E or H. This was thought to be because the lower nitrogen

content of the diet supported a less numerous, and hence less active, microbial population in the rumen. This is an example of the dangers of relying on *in vitro* tests to predict behaviour *in vivo*, where the substrate tested is very different from the diet of the animal used to supply the rumen liquor inoculum.

Table 5. *Daily rations fed to sheep*

Constituent	Quantities fed (g./day)			
	Diet E	Diet H	Diet G	Standard*
Hay	600	600	600	300
Flaked maize	—	—	—	300
Concentrates 411†	150	150	150	—
Groundnut meal E‡	100	—	—	—
Herring meal	—	130	—	—
Groundnut meal	—	—	30	300
Total N content	19	23	12	30

In diets E, H and G the hay was fed in two lots at 11.00 and 16.00 hr., the remainder at 07.00 hr.; half the standard ration was fed at 07.30 hr., half at 19.30 hr.

* Diet fed to sheep used to supply rumen liquor inoculum for preliminary *in vitro* tests.

† 4 parts ground maize, 1 part wheat bran, 1 part crushed oats.

‡ A special groundnut meal, solvent-extracted under harsh conditions and of low salt peptizability, supplied by the Research Department of J. Bibby and Sons Ltd., Liverpool.

Fig. 4

Fig. 5

Fig. 4. Ammonia production in the rumen of sheep 377 from the three rations; ●, E; ○, H; +, G.

Fig. 5. Ammonia production in the rumen of sheep 416 from the three rations; ●, E; ○, H; +, G.

DISCUSSION

For convenience in the calculations above it was assumed that the volumes of the rumen contents of the sheep used were of the order of 5 l. This is probably an underestimate for the larger animals, but minor discrepancies would not affect the argument.

In the present state of knowledge of the biochemical and microbiological events in the rumen, there seem to be no grounds for relying on one or a few criteria of normal rumen function; rather as large a number and variety of criteria as possible should be used. While biological criteria are probably simpler to apply, they have more value in long-term investigations than in work lasting no more than a few hours. On the other hand, the chemical

criteria such as the rates of digestion of substrate or of production of metabolite can be quite sensitive over short periods of time. In this connexion, the expression of results in units such as g./l./hr. or day, rather than as digestibilities or other relative values, is to be recommended as making comparisons between different techniques simpler.

The latter part of the last experiment shows that not only must the physical environment in an artificial rumen approximate to normal (maintenance of a suitable temperature, pH value, gas phase, provision for removal of metabolites) but also the substrates tested must approximate in nature and quantity to the diet of the animal from which the rumen liquor inoculum was taken, if the *in vitro* results are to serve as indications of results *in vivo*. In other cases the *in vitro* results may serve to show what might happen *in vivo* on first changing the ration of the animal, but it is well known that several days or weeks are needed for the rumen microbial population to adapt to a new diet, behaviour during the first few days following a change of diet being relatively unpredictable.

The too frequent practice of adding *in vitro* to rumen liquor taken from an animal on some standard diet, a single dose of some substance or mixture unrelated to the diet, incubating for lengthy periods of time and using the results to attempt to predict the effects of feeding that substance or mixture in addition to or even instead of the standard diet, with little or no attempt to show that the *in vitro* system used successfully reproduces the features of rumen function *in vivo*, is to be deprecated.

The work reported here was supported by a grant from the Agricultural Research Council; most of it was incorporated in a Dissertation submitted (1955) to the University of Aberdeen for the degree of Ph.D.

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(Received 2 January 1956)