

Use of particle-bound microbial enzyme activity to predict the rate and extent of fibre degradation in the rumen

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1. A method was developed for extracting enzymes from micro-organisms closely associated with ammonia-treated straw ($\text{NH}_3\text{-S}$) that had been incubated in nylon bags in the rumen. Incubation of washed straw with 125 ml carbon tetrachloride/l and 20 μg lysozyme/ml for 3 h at 37 ° gave carboxymethylcellulase (EC 3. 2. 1. 4; CMCase) and NAD-linked glutamate dehydrogenase (EC 1. 4. 1. 2; GDH) activities greater than those extracted by sonication.

2. GDH associated with $\text{NH}_3\text{-S}$ increased with incubation time and was highest in sheep receiving a high-barley diet. Particle-bound CMCase activity reached a peak between 16 and 24 h and declined thereafter.

3. Particle-bound GDH activity showed no correlation with dry matter (DM) degradation in the rumens of sheep fed on a range of diets. In contrast, CMCase activity after 24 h was highly correlated with DM degradability of the same samples at 24 h (r 0.98) and 48 h (r 0.94).

4. It was concluded that GDH and CMCase can be used as indices of the total population of colonizing rumen micro-organisms and of the fibre-degrading population respectively, and that these enzymes can therefore be used to assess rapidly and with great sensitivity variations in the rumen environment that affect the rate of fibre breakdown.

Most (50–70%) bacteria in the rumen are associated with food particles (Warner, 1962; Minato *et al.* 1966), and 75% of the rumen microbial adenosine-5'-triphosphate is particle-bound (Forsberg & Lam, 1977). This microbial population is closely associated with the feed particles and is directly responsible for their degradation. The cellulolytic bacteria that are important in forage degradation adhere closely to plant cell wall material, forming distinctive digestive pits on the cell walls (Akin, 1979; Cheng *et al.* 1981). When roughages are treated with alkali the resultant increase in the rate and extent of degradation is associated with a massive increase in the numbers of colonizing cellulolytic bacteria (Latham *et al.* 1979).

These observations led to the possibility that a measurement of colonization could provide a basis for predicting the rate and extent of forage degradation. If such a test could be applied, it would be possible to screen a large number of samples in a short time, as colonization could potentially be studied after a brief incubation period. A measurement based on colonization might also detect with greater sensitivity changes in the rumen flora and their activities that arise as a result of changes in the rumen environment. The aim of the present study was therefore to estimate the degree of microbial colonization of particulate material from the rumen by measuring the activities of particle-bound microbial enzymes, and to compare the rate and extent of colonization with the rate of loss of dry matter (DM) from nylon bags suspended in the rumen. A preliminary report of this work has been published (Silva *et al.* 1985).

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MATERIALS AND METHODS

Experimental animals and diets

Suffolk cross sheep were used. They were fitted with 50-mm rumen cannulas while under general anaesthesia, taking normal aseptic precautions. For Expt 1, a castrated male sheep weighing 50 kg and receiving ammonia-treated straw ($\text{NH}_3\text{-S}$) *ad lib.* was used. For Expt 2, two castrated male sheep weighing 45 and 60 kg were used, one receiving 800 g/d of a diet consisting of (g/kg) 900 whole barley and 100 fish meal (FM), the other a diet similar to that used in Expt 1. For Expt 3, eight castrated males and four females weighing 45–75 kg were used. They were allocated at random to each of the following four diets with three receiving each diet (g/kg): A, whole barley 900, FM 100; B, whole barley 500, straw 500; C, all straw diet; D, straw 850, sugar-beet pulp (without added molasses; SBP) 150. Diet A was given at 0.8 kg/d while the other diets were offered *ad lib.* For Expt 4, eight castrated male sheep weighing 45–60 kg were used. They were allocated randomly to each of the following four diets with two receiving each diet (g/kg): A, rolled barley 900, FM 100; B, rolled barley 600, hay 400; C, molasses 400, hay 600; D, all hay diet. Hay, straw and $\text{NH}_3\text{-S}$ used in all the experiments were hammer milled using a bale grinder with a 40 mm screen, and urea and a mineral–vitamin mix were added according to Agricultural Research Council (1980) recommendations.

The sheep were fed twice daily at 09.00 and 16.00 hours. Animals were penned individually on wooden slatted floors and were given free access to water. In all the experiments, an adaptation period of 3 weeks was allowed before measurements were made.

Nylon-bag incubations

Bags were made from woven nylon cloth, pores 20–40 μm , and were 120 \times 80 mm in size. Approximately 2 g air-dry $\text{NH}_3\text{-S}$, ground using a hammer mill with a bar screen (4-mm bars, 3-mm gaps), were weighed into each bag. Bags were attached to slits on PVC tubing anchored to the rumen cannula top. More than one batch of $\text{NH}_3\text{-S}$ was used in these experiments, so direct comparisons cannot be made between different experiments. Nylon-bag incubations for DM degradation were carried out according to Ørskov *et al.* (1980). Bags for enzyme activity measurements and DM degradation were incubated simultaneously.

Immediately after withdrawal from the rumen, bags were washed in cold water in a household washing machine (Indesit model 2550). The length of the washing program finally selected was 13 min, consisting of three rinsing cycles of 1 min 25 s each. After washing, the bags for DM determinations were dried at 60° for 48 h. The bags used for enzyme activity measurements were either frozen at -20° or assayed immediately.

Extraction of enzyme activity

Initially, enzyme activity was extracted from particulate matter by sonication. Bags removed from the rumen were washed and then squeezed thoroughly and the contents were mixed with 50 ml 20 mM-Tris hydrochloride buffer, pH 7.0. The organisms attached to fibres were disrupted by sonication at 5A, 0° for ten 30 s periods, with intervening 30 s intervals to allow cooling. Fibres were removed and the filtrate was centrifuged at 29000 g at 4° for 15 min. The supernatant fluid was used to estimate NAD-linked glutamate dehydrogenase (EC 1.4.1.2; GDH) activity.

Subsequently, a chemical extraction procedure similar to that of Nossal & Heppel (1966) was used. Undegraded residue (1 g wet weight) from the washed nylon bags was mixed with 20 ml 10 mM-sodium phosphate (pH 6.8) buffer and was treated with 2.5 ml carbon tetrachloride and 20 μg lysozyme/ml at 37° for 3 h. Fibres and particulate material were

removed as for the sonicated extract. Using another 1 g from each bag, the DM content of the undegraded residue was determined.

Enzyme activities of rumen fluid were measured following sonication. Samples were strained through two layers of muslin, and 1 ml filtrate was mixed with 24 ml buffer, sonicated and centrifuged as before.

Enzyme assays

Particle-bound GDH activity was determined from the rate of oxidation of NADH in the presence of 2-oxoglutarate (Meers *et al.* 1970). The rate of NADH oxidation was calculated as $\mu\text{mol}/\text{min}$ per ml, and was adjusted for total volume of the buffer to give GDH activity expressed as $\mu\text{mol}/\text{g DM}$ per min.

Carboxymethylcellulase (EC 3.2.1.4; CMCase) was measured using the method described by Groleau & Forsberg (1981). Hydrolysis of sodium carboxymethylcellulose (Sigma Chemical Co. Ltd, Poole, Dorset) was assayed by measuring the formation of reducing sugar using 3,5-dinitrosalicylic acid (DNS) reagent (Miller *et al.* 1960). D-Glucose was used as a standard. CMCase activity was expressed as μmol glucose released/g DM per min.

Protein in rumen fluid was determined using the Folin reagent (Herbert *et al.* 1971).

Statistical analysis

The statistical significances of differences between the means was assessed by Student's *t* test. Correlation coefficients were calculated for DM degradability of $\text{NH}_3\text{-S}$ and particle-bound CMCase activity at each incubation period. DM degradability values were analysed by analysis of variance.

RESULTS

Extraction of enzyme activity

The first step in the preparation of particle-bound enzymes was the removal of non-adherent micro-organisms by washing. The efficiency of the washing procedure was determined by washing, for different periods, bags that had been incubated for 8 h in the rumen, and then extracting enzymes by sonication (Expt 1; Fig. 1). GDH and CMCase activities and the variation observed between samples gradually declined with increasing washing time. The variation among the samples at the end of the first rinsing cycle for CMCase was 137.3 μmol glucose released/g DM per min between the highest and lowest activities, compared with that of 14.5 μmol glucose released/g DM per min at the end of the complete programme. Equivalent values for GDH were 4.1 and 1.4 μmol NADH oxidized/g DM per min. Washing the samples for a further 5 min period in cold water did not reduce the variation, but the activity was slightly decreased. A cycle consisting of three rinses was decided on for subsequent experiments.

Various methods for chemical extraction of enzymes were investigated (Table 1) to find an alternative to the efficient but time-consuming sonication procedure. Extraction with chloroform, lysozyme and EDTA in Tris-hydrochloride buffer, pH 7.0, for 10 min released only 7–8% of the total activity obtained with sonication. Up to 26% of CMCase activity was released after 1 h in the presence of CCl_4 , and a complete recovery of the enzyme activity was achieved when the incubation time was increased to 3 h and EDTA was omitted. Incubating the sample with 100 mM-citrate buffer (pH 5.0) led to a very low activity.

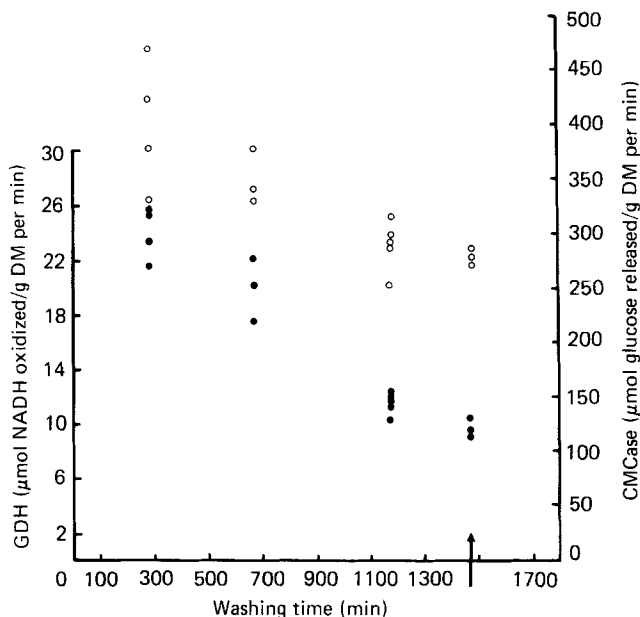


Fig. 1. Expt 1. Effect of washing time on particle-bound glutamate dehydrogenase (*EC* 1.4.1.2; GDH) (●) and carboxymethylcellulase (*EC* 3.2.1.4; CMCase) (○) activities associated with ammonia-treated straw (NH₃-S) incubated for 8 h in the rumen of a sheep receiving NH₃-S. For details of procedures, see p. 408. DM, dry matter; †, machine wash plus 5 min in cold water.

Table 1. *Extraction of enzyme activities from particle-bound micro-organisms*

(Ammonia-treated straw (NH₃-S) was incubated for 24 h in nylon bags in sheep receiving NH₃-S *ad lib.*, was washed and enzymes were extracted by sonication. Various alternative extraction procedures were investigated in NH₃-S from parallel incubations. Carboxymethylcellulase (*EC* 3.2.1.4; CMCase) is expressed in µmol glucose released/g DM per min, and glutamate dehydrogenase (*EC* 1.4.1.2; GDH) in µmol NADH oxidized/g DM per min. Values are means and standard deviations, *n* 6)

Treatment	Activity released			
	CMCase		GDH	
	Mean	SD	Mean	SD
Sonication	975	44	23.1	4.6
125 ml carbon tetrachloride/l, 20 µg lysozyme/ml, 40 mM-EDTA in 20 mM-Tris hydrochloride, pH 7.0:				
37°, 10 min	75	76	—	—
37°, 1 h	255	72	—	—
125 ml CCl ₄ /l, 20 µg lysozyme/ml in:				
20 mM-Tris hydrochloride, pH 7.0, 37°, 3 h	953	45	—	—
10 mM-sodium phosphate, pH 6.8, 37°, 3 h	1015	51	24.7	10.1
100 mM-sodium citrate, pH 5.0, 37°, 3 h	88	9	—	—

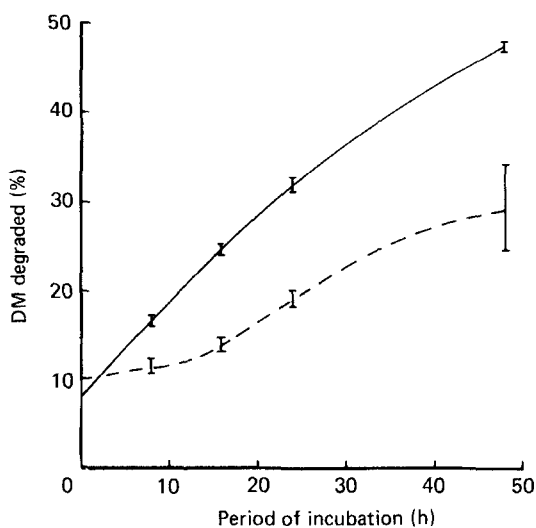


Fig. 2. Expt 2. Dry matter (DM) degradation of ammonia-treated straw incubated in two sheep fed on ammonia-treated straw (—) or barley + fish meal (---). Values are means and standard deviations represented by vertical bars, for four determinations. For details of diets, see p. 408.

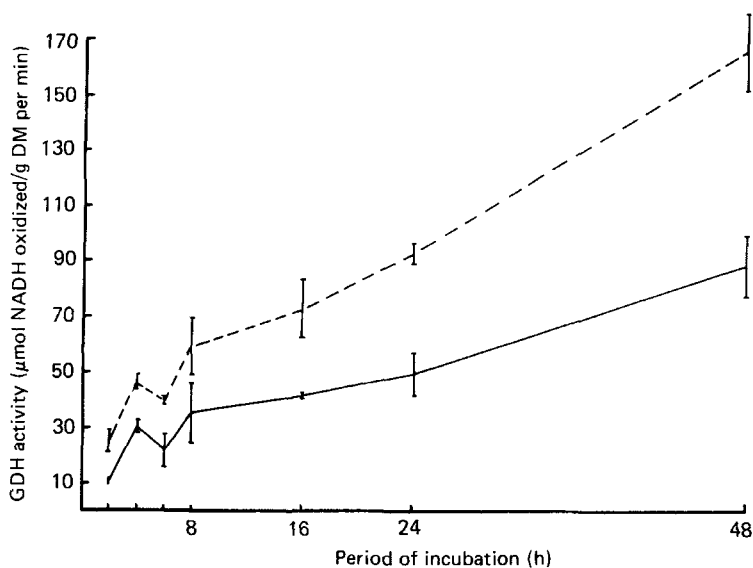


Fig. 3. Expt 2. Particle-bound glutamate dehydrogenase (*EC* 1.4.1.2; GDH) activity of ammonia-treated straw incubated in two sheep fed on straw alone (—) or barley + fish meal (---). DM, dry matter. Values are means and standard deviations represented by vertical bars for four determinations. For details of diets, see p. 408.

Relation between DM degradation, particle-bound CMCase and GDH activities and incubation time in situ

The DM degradability of $\text{NH}_3\text{-S}$ was much lower in the sheep fed on a barley diet than in those receiving $\text{NH}_3\text{-S}$ (Expt 2; Fig. 2). The difference between the two animals became apparent after an 8 h incubation period and remained up to 48 h. In contrast the GDH activity showed a completely reversed pattern (Fig. 3). $\text{NH}_3\text{-S}$ incubated in the animal fed

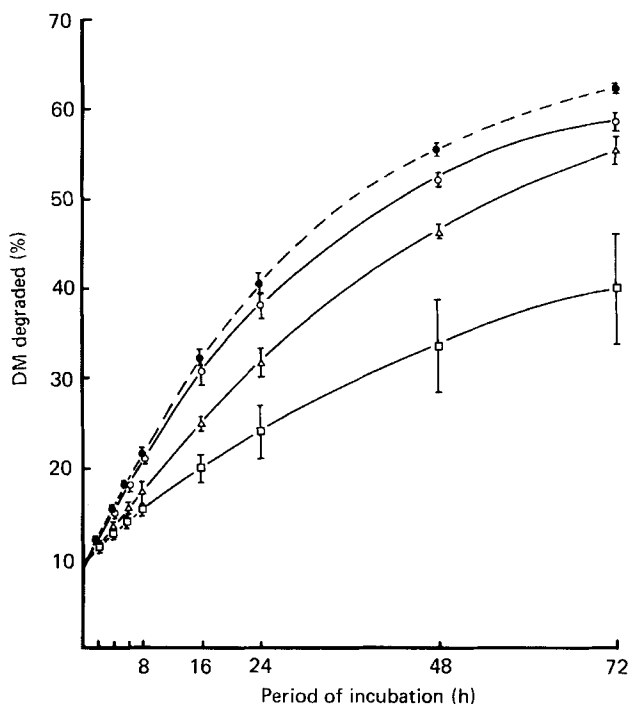


Fig. 4. Expt 3. Dry matter (DM) degradation of ammonia-treated straw incubated in sheep fed on barley + fish meal (\square), straw + barley (\triangle), straw (\circ) or straw + sugar-beet pulp (\bullet). Values are means and standard deviations represented by vertical bars for six determinations. For details of diets, see p. 408.

on a high-barley diet had a high particle-bound GDH activity throughout the incubation period. An initial rise in GDH activity was observed in both animals from 2 to 4 h, followed by a decrease at 6 h. The differences were not significant at 6 and 8 h. Except for these two incubation periods the GDH activity of incubated straw was significantly higher in the animal fed on the high-barley diet.

The GDH activity of rumen fluid was also measured in the animals used in Expt 2. The straw-fed sheep had an activity of 21.9 (SE 3.0, n 4) $\mu\text{mol/ml}$ per min, compared with 58.0 (SE 16.2, n 4) $\mu\text{mol/ml}$ per min for the sheep receiving barley + FM. However, this difference was accompanied by a difference in the protein content of the rumen fluids, with 8.5 (SE 0.3) and 21.9 (SE 3.0) mg/ml being present in the respective animals, leading to similar specific activities of 2.34 (SE 0.35) and 2.63 (SE 0.38) $\mu\text{mol/mg}$ protein per min in the two sheep.

In a separate experiment, the development of particle-bound CMCCase activity was followed in sheep fed on different diets, and was compared with DM degradation (Expt 3). Again, the rate of degradation of $\text{NH}_3\text{-S}$ was slowest in the sheep receiving barley (Fig. 4). However, in contrast to GDH, CMCCase activity was lowest in these sheep, and highest in sheep receiving straw + SBP (Fig. 5). Also, unlike GDH, the particle-bound activity peaked between 16 and 24 h, and declined thereafter until activities were similar at 72 h (Fig. 5). The correlation between DM degradation at 48 h and CMCCase activity associated with $\text{NH}_3\text{-S}$ was significant ($P < 0.01$) in samples removed at 8, 16, 24 and 48 h, with the 24 h sample having the highest correlation (r 0.98).

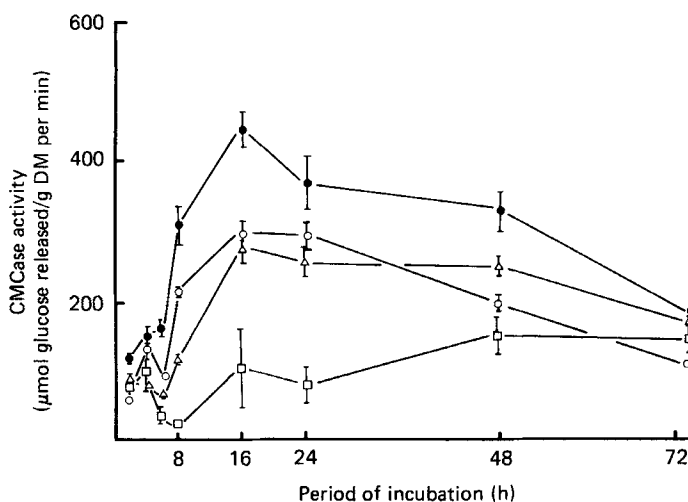


Fig. 5. Expt 3. Particle-bound carboxymethylcellulase (*EC* 3.2.1.4; CMCCase) activity of ammonia-treated straw incubated in the rumen of sheep fed on barley + fish meal (□), straw + barley (△), straw (○) or straw + sugar-beet pulp (●). DM, dry matter. Values are means and standard deviations represented by vertical bars for six determinations. For details of diets, see p. 408.

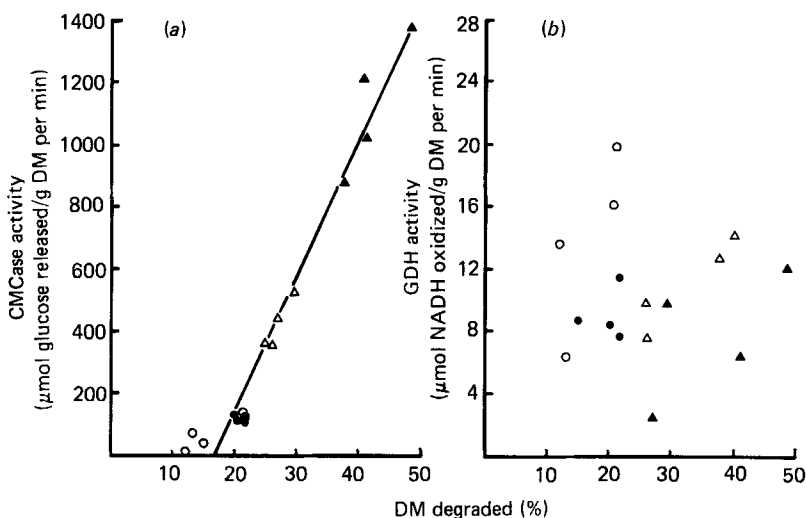


Fig. 6. Expt 4. Relation between (a) carboxymethylcellulase (*EC* 3.2.1.4; CMCCase) and (b) glutamate dehydrogenase (*EC* 1.4.1.2; GDH) and dry matter (DM) degraded after 24 h incubation in sheep fed on barley + fish meal (○), barley + hay (●), hay + molasses (△) and hay alone (▲). For details of diets, see p. 408.

Relation between DM degradation and particulate microbial CMCCase and GDH activities in individual sheep fed on four different diets

In Expt 4, both CMCCase and GDH activities were compared with DM degradability in individual animals receiving four different diets. There was a wide variation in degradability at 24 h and 48 h between the diets, and a corresponding variation in CMCCase activity at the same times, with a correlation coefficient of 0.98 at 24 h (Fig. 6(a)) and 0.85 at 48 h (not shown). The correlation coefficient relating CMCCase activity at 24 h with DM degradation at 48 h was 0.94 (not shown). The highest CMCCase at 24 h was found in hay-fed

sheep (1143 $\mu\text{mol/g DM per min}$), with a mean DM degradability of 0.416, and the lowest values were found in sheep receiving barley+FM, (68 $\mu\text{mol/g DM per min}$ and 0.181 respectively). Extracted GDH activities bore no relation to DM digestibility at 24 h (Fig. 6(b)) or 48 h (not shown).

DISCUSSION

The work described here was an attempt to investigate the possibility of using particle-bound enzymes to assess microbial colonization as a measure of the rate and extent of forage degradation. In order to achieve this, methods were developed for the removal of contaminating, non-adherent micro-organisms from fibres and for the extraction of enzymes from the adherent population. Washing for less than 2 min in a domestic washing machine was not sufficient to remove loosely adherent and trapped organisms from $\text{NH}_3\text{-S}$ that had been incubated in the rumen in nylon bags (Fig. 1). Longer washing periods did produce more consistent values, although a constant asymptote was never reached. The finding that after a certain extent of washing further removal of organisms was difficult is in accord with Minato *et al.* (1966) and Williams & Strachan (1984). In both these experiments even washing digesta residues with surfactants failed to remove the most tightly adherent bacteria. Diaminopimelic acid measurements showed 10% residual bacterial matter after washing digesta solids with a Tween 80 buffer for 1 h (Williams & Strachan, 1984).

GDH is found in both cellulolytic and non-cellulolytic bacteria (Joyner & Baldwin, 1966), so it may be assumed that extracted GDH reflects the total extent of microbial colonization. CMCase activity, on the other hand, is presumably restricted to micro-organisms intimately involved in fibre degradation. Therefore the pattern of removal of the different enzymes during washing (Fig. 1), with GDH activity declining more quickly than CMCase, indicates a preferential retention of fibre digesters. The final washing procedure adopted was a compromise between the need to remove contaminating non-cellulolytic micro-organisms and the retention of the micro-organisms of principal interest.

It was clear from the GDH measurements that the total number of bacteria attached to $\text{NH}_3\text{-S}$ had no relation to their ability to degrade the substrate. More colonization occurred in the high-barley-fed sheep than in the sheep receiving $\text{NH}_3\text{-S}$ and this was proportional to the microbial biomass present in the rumen fluid. Thus GDH appeared to measure the extent of non-specific attachment. GDH was therefore a poor indicator of fibre degradation (Fig. 6). Particle-bound GDH activity gradually increased with advancing incubation periods, indicating a progressive microbial colonization. A similar observation has been made by Mathers & Aitchison (1981) in which ^{35}S incorporation into rumen microbes indicated a continuous increase in microbial protein in nylon-bag residues with increasing residence time in the rumen.

Cellulolytic activity as measured by CMCase was, in contrast, a good indicator of DM degradation. A high correlation was seen between CMCase activity and DM degradability of $\text{NH}_3\text{-S}$ in different rumen environments (Fig. 6). Therefore, the key factor governing degradation is not a higher microbial density but the establishment of a specific, CMCase-containing population. The extent to which nutritional factors such as the supply of nitrogen, minerals and vitamins and resultant rumen conditions, such as pH, could affect fibre digestion would be assessed better by measuring the effect of these factors on adherent CMCase activity than by measuring total colonization.

Re-calculation of the values shown in Fig. 4 to show DM disappearance between the various incubation times produces a pattern (Fig. 7) rather similar to that of CMCase activity (Fig. 5). For example the sharp rise in DM degraded from 8 to 16 h seen in an animal

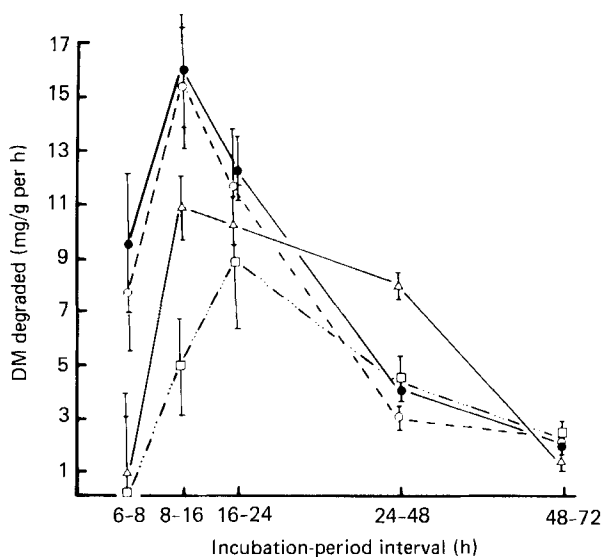


Fig. 7. Expt 3. Degradation of dry matter (DM) during various time-periods in sheep fed on barley + fish meal (□), straw + barley (△), straw (○) or straw + sugar-beet pulp (●). These values were obtained from Fig. 4. Values are means and standard deviations represented by vertical bars for six determinations. For details of diets, see p. 408.

given straw with 500 g barley/kg in Fig. 4 was reflected in a rapid increase in particle-bound CMCase activity in the same period (Fig. 5). The particle-bound CMCase activity therefore not only reflects the final degradability but accentuates the small differences observed in the rate of degradation that are important in determining food intake, etc. The method thus achieves a higher sensitivity than a time-related DM degradability measurement in assessing differences between different rumen environments, and could potentially be used to study the effects of different treatments on altering the degradability of the cellulosic substrate.

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