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XYLANASE (HEMICELLULASE) ACTIVITY IN CELL-FREE RUMEN FLUID

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ABSTRACT

It has been shown that there is hemicellulase (xylanase) activity in cell-free filtrates of rumen liquor. This activity changes during the feeding cycle. The optimal pH and temperature for this activity have been found, as have the substrate-to-enzyme ratios. Many reagents, particularly heavy metal ions and phenols, inhibit the activity, but the activity is enhanced by reducing agents. No activity towards monosaccharides, disaccharides, or glycosides was found. The xylanase component was not stable, due to proteolytic enzymes in the rumen liquor, but could be purified by a variety of methods to give more-stable enzymes.

INTRODUCTION

The structural carbohydrates of the plant cell-wall are an important source of energy to ruminant animals, since they can be degraded by certain species of rumen bacteria into products that can be absorbed and metabolized by the host animal. The species of bacteria that can utilize cellulose and the hemicelluloses are well-characterized[1–3], but there is conflicting evidence as to the site of degradation. Howard *et al.*[4] found only traces of xylanase activity in cell-free filtrates of cultures of rumen bacteria, but found high xylanase activity, as well as xylobiase activity, in the supernatant solution obtained after disruption of the bacterial pellet. Beveridge and Richards[5] reported that ~30% of an isolated hemicellulose was converted into reducing sugars in 72 h by incubation with enzymes produced by disrupting the bacterial cells, whereas, under the same conditions, only ~3% was converted into reducing sugar with cell-free rumen fluid. On the other hand, Dehority[6] claimed that up to 64% of a purified xylan could be degraded by cell-free filtrates of cultures of rumen bacteria. Similarly conflicting evidence on the site of breakdown of cellulose in the rumen is present in the literature. Kitts and Underkofler[7] stated that cellulolytic enzymes are not present as such in rumen fluid, whereas Gill and King[8] found at least three cellulases that were free in rumen fluid.

The object of the present study, which is part of a project aimed at improving the utilisation of plant materials by ruminants, was to investigate the site of xylan

degradation in the rumen, to determine the rate of xylan degradation, and to examine methods of purifying the enzymes.

EXPERIMENTAL

Animals and diets. — Two wethers, fitted with rumen cannulas, were kept on a daily diet of 1.2 kg of hay given in two equal feeds at 0900 and 1600 h. Samples of rumen contents were withdrawn as required (usually before the morning feed) and filtered twice through muslin.

Materials and analyses. — All reagents, and most of the carbohydrates, used were obtained from commercial sources and were of the highest purity. A hemicellulose preparation was obtained from the hay used in the diet (*Lolium perenne*: S23) by first delignifying the sample by the acetic acid–sodium chlorite method[9], followed by extraction of the hemicelluloses with M potassium hydroxide. The hemicelluloses were precipitated and dried as previously described[10], and fractionated by the iodine-precipitation method[11] to give a "linear" and a "branched" xylan. Total carbohydrate was determined by the phenol–sulphuric acid method[12]. Reducing sugars were determined by the method of Somogyi[13]. Protein was determined by the method of Lowry *et al.*[14]. Molecular-sieve chromatography was carried out on columns of either Sephadex gels (Pharmacia Ltd., London) or Corning controlled-pore glass granules (B.D.H., Poole). Columns were eluted with water, and the eluant monitored continuously at 280 nm.

Incubations. — Strained rumen contents were centrifuged at 4° and 100 *g* for 20 min to remove large food-particles and protozoa. The supernatant solution was recentrifuged at 4° and 50,000 *g* for 30 min to remove bacteria[15]. On examination under the microscope, the supernatant was found to be essentially free from bacteria. The cell-free rumen fluid (1 ml) was mixed with polysaccharide solution (0.1%, 1 ml) and water (1 ml). To this was added dithiothreitol solution (0.2%, 1 ml), in order to maintain the enzyme in the reduced form. The mixture was incubated at 37° for 18 h. The time between preparation of the cell-free rumen fluid and the start of the incubations was kept to a minimum because of loss of activity in the rumen liquor if left too long.

RESULTS

Diurnal variation in xylanase activity. — Rumen liquor was removed from a sheep at 0900 h (pre-feed) and subsequently at hourly intervals throughout the day until 1600 h. Cell-free fluid was prepared and the incubations were carried out as described above, except that the concentration of the polysaccharide solution was varied between 0.02 and 0.2% in different experiments. Different hemicellulose preparations were also used, but all incubations were carried out for the same period of time. To prevent any deterioration, the incubations were started as soon as the cell-free fluid was prepared. The results of several experiments are shown in Fig. 1.

When the activity was low (determined by the increase in reducing sugar content), there appeared to be little change during the day, but when the activity was high there appeared to be a slight decrease in xylanase activity immediately after feeding, followed by a rise in activity between 3 and 5 h after feeding. By the time the next feed was due, the activity had returned to the pre-feed values.

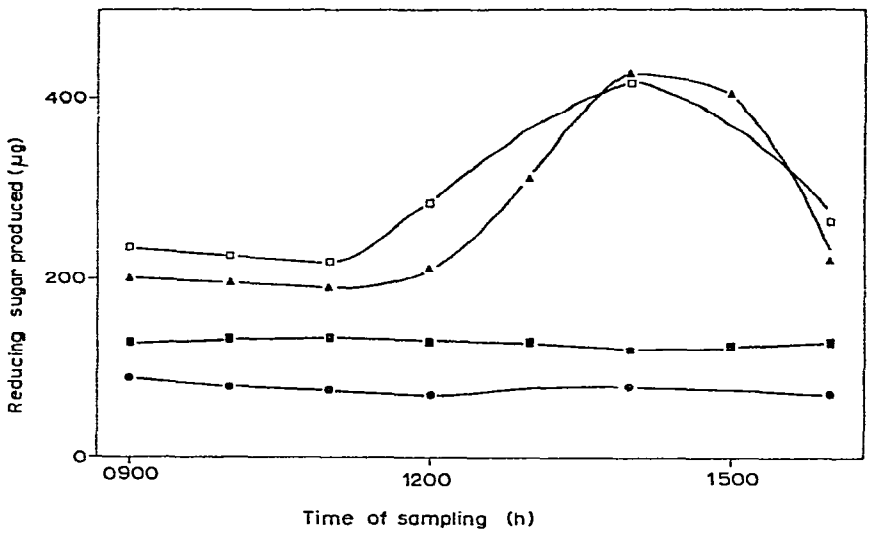

Fig. 1. Diurnal variation in xylanase activity with different substrates at different concentrations: □——□, linear xylan at 1 mg per ml; ■——■, linear xylan at 0.5 mg per ml; ▲——▲, branched xylan at 1 mg per ml; ●——●, branched xylan at 0.5 mg per ml.

Effect of altering the rumen fluid:polysaccharide ratio. — Incubations were carried out where (*a*) the concentrations of cell-free rumen fluid were kept constant and the quantity of polysaccharide used was varied, and (*b*) the amount of polysaccharide was kept constant and the concentration of cell-free rumen fluid was varied. Typical results are shown in Figs. 2 and 3, respectively, and show that, in an incubation mixture (4 ml) containing 500–2000 μg of substrate and 25–50% cell-free rumen fluid, first-order reaction kinetics were followed. The amount of polysaccharide hydrolysed was dependent on the source and composition of the substrate. In the two examples shown in Fig. 2, the conversion values for incubation times of 18 h were 0.24 and 0.17, respectively, for the two samples. The conversion value is defined as the amount of reducing sugar produced on incubation divided by the amount of reducing sugar that would be produced if the polysaccharide was completely hydrolysed.

Other carbohydrase activities. — The glycosidase activity of cell-free rumen fluid was determined against the following substrates: methyl α- and β-D-glucopyranosides, methyl α- and β-D-galactopyranosides, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, methyl β-D-arabinopyranoside, maltose, isomaltose,

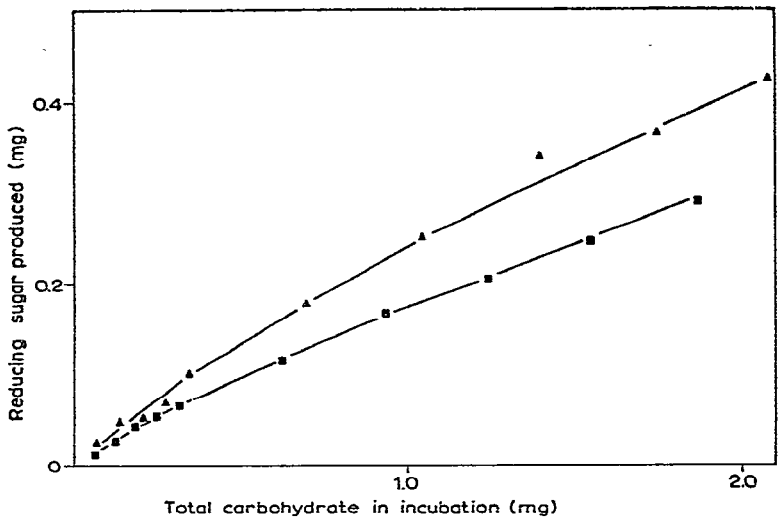

Fig. 2. Percentage hydrolysis of xylans when the rumen fluid concentrations are kept constant and the xylan concentrations are varied. ▲——▲, linear xylan; ■——■, branched xylan.

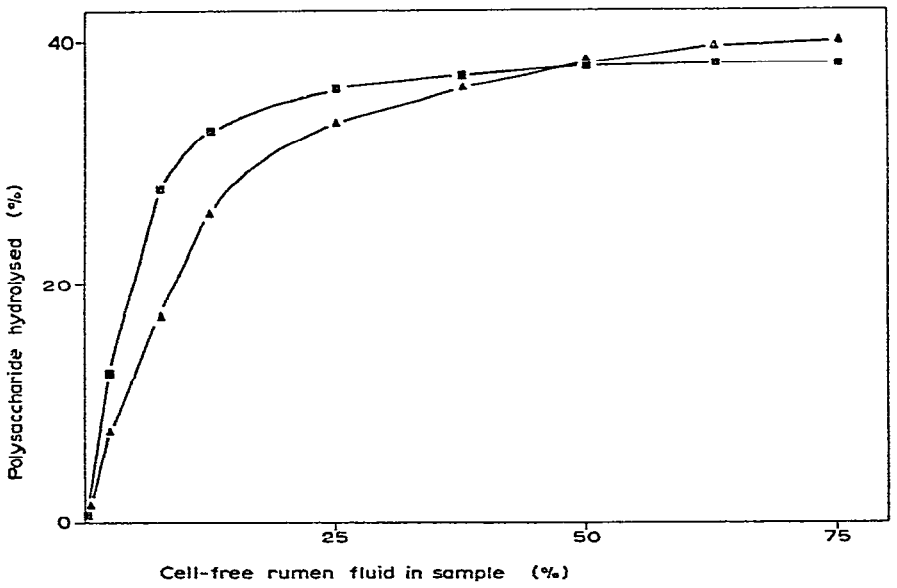

Fig. 3. Percentage hydrolysis of xylans when the rumen fluid concentrations are varied and the xylan concentrations are kept constant: ▲——▲, linear xylan; ■——■, branched xylan.

cellobiose, gentiobiose, melibiose, and lactose. There was no increase in the reducing-sugar content of any samples after incubation, indicating that cell-free rumen fluid did not contain any of the glycosidases necessary to hydrolyse those substrates. At the same time, no decrease in total carbohydrate was found, indicating that none of the sugars was undergoing glycolysis.

Effect of pH on xylanase activity. — Cell-free rumen fluid was prepared as above, and 15 2-ml aliquots were transferred to 10-ml volumetric flasks that contained xylan substrate. Quantities of hydrochloric acid and sodium hydroxide were added to the flasks and they were made up to the mark with water. The quantities of acid and alkali used were such that the final pH of the incubations lay between 2 and 11, with most samples being between 5.5 and 7.5. The samples were incubated as described above and the reducing content determined. The results are shown in Fig. 4. The amount of reducing sugar produced was at a maximum at pH 7.05. Above that value, the activity fell away rapidly but only reached zero at pH 11. Below pH 7, the activity dropped only slightly until below pH 5.5, when it fell very rapidly to a constant, but positive, value.

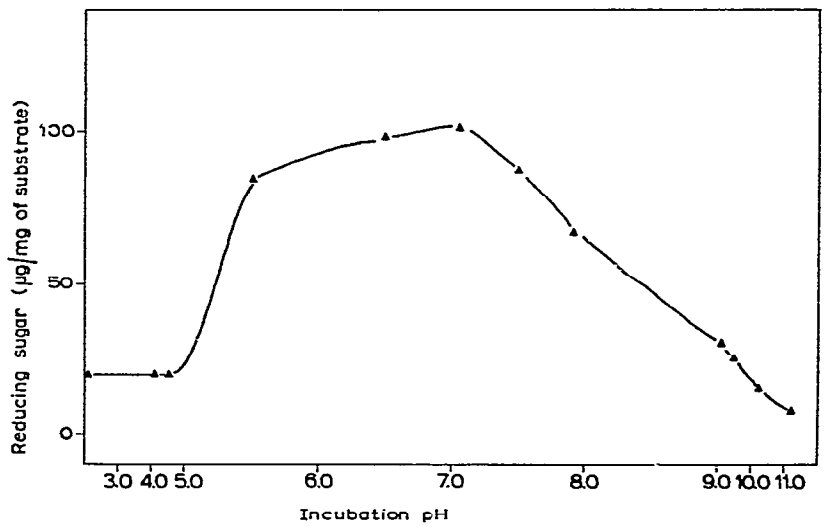

Fig. 4. Effect of pH on cell-free rumen-fluid xylanase activity.

Effect of incubation temperature on xylanase activity. — Cell-free rumen fluid was prepared as described above and incubated with xylan substrate, in triplicate, as already described at various temperatures between 4 and 65°. The increase in reducing sugar content was determined, and the results are shown in Fig. 5 for two xylan preparations. The maximal activity appeared at 31°, not at the rumen temperature of 39°. The activity was high between 20 and 45°, but declined more sharply above 45° and below 20°, Even at 4°, considerable xylanase activity was present.

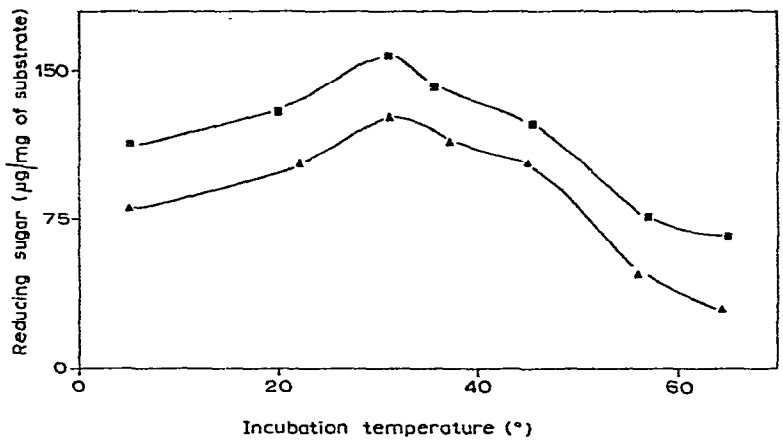

Fig. 5. Effect of incubation temperature on cell-free rumen-fluid xylanase activity: ■——■, linear xylan; ▲——▲, branched xylan.

Stability of xylanase activity at different temperatures. — Cell-free rumen fluid, prepared as above, was maintained at 4°, 20°, or 37° for 6 h. Every hour, aliquots were removed and incubated with a xylan substrate by the standard incubation procedure. The samples were all incubated for the same length of time at 37°, and the increase in the reducing sugar content of the incubations was determined. At all three temperatures, there was a fairly rapid decrease in activity during the first two hours, but from then on the activity declined at a lower rate. However, even after six hours at each temperature, the activity was still dropping. The decrease in xylanase activity was greatest at 37° and least at 4°. After six hours at the three temperatures, the activity of the 37°-sample was 43% of the original value, that of the 20°-sample was 55%, and that of the 4°-sample was 81%.

Effect of other carbohydrates on the xylanase activity. — When cell-free rumen fluid was incubated with xylan substrate in the presence of D-arabinose, L-arabinose, D-xylose, D-mannose, D-galactose, or D-glucose up to concentrations of 250 µg/ml, there was no effect on the xylanase activity. Incubation with sucrose, lactose, maltose, and cellobiose, in the same range of 0–250 mg/ml, also had no effect on the xylanase activity, except for cellobiose at the highest concentration which caused slight inhibition. When cell-free rumen fluid and a xylan substrate were incubated in the presence of cellulose, there was an "apparent" loss of activity over the control, as measured by the increase in reducing sugar content. However, there was an equivalent decrease in the concentration of the total carbohydrate in solution, even though the protein content of the solution remained constant.

Effect of various salts and other reagents on xylanase activity. — The effect of certain ions and other reagents that could cause inhibition of enzyme activity was investigated by incorporating these reagents in the standard incubation mixtures and then determining the change in reducing sugar content, relative to a reagent blank.

The reagents were incorporated at two or three concentrations. The results are shown in Table I. Space does not allow a complete analysis of the data, but as can be seen from Table 1, the reagents have been grouped into four categories. There is also a maximal concentration used beside each reagent. The four categories were, no effect, slight (0–20%) inhibition, strong (>20%) inhibition, and activation. Most common ions had no effect on the enzyme activity, whereas heavy metals caused almost complete inhibition. Phenolic compounds caused slight inhibition, whereas reducing agents caused an increase in the enzyme activity; the greatest increase in activity was obtained using dithiothreitol as the reducing agent. The increase in activity, although not great, was ~10% at a final concentration of 0.05% of dithiothreitol.

TABLE I

EFFECT OF VARIOUS SALTS AND OTHER REAGENTS ON XYLANASE ACTIVITY[a]

No effect	*Slight inhibition*	*Strong inhibition*	*Activation*
Na^+	Phenol (0.05M)	Zn^{2+} (0.01M)	Dithiothreitol (5mM)
K^+	Thymol (0.05M)	Fe^{3+} (0.01M)	Fe^{2+} (0.1M)
Ca^{2+}	$S_2O_3^{2-}$ (0.1M)	Co^{2+} (0.01M)	SO_3^{2-} (0.1M)
Mg^{2+}	Phenylmercuric	Cu^{2+} (0.01M)	
Cl^-	acetate (0.2mM)	Mn^{2+} (0.01M)	
F^-		CO_3^{2-} (0.1M)	
OAc^-		*N*-Bromosuccinimide	
SO_4^{2-}		(0.001M)	
$H_2PO_4^-$			
HPO_4^{2-}			
HCO_3^-			
EDTA			
Me_2SO			
(all 0.1M)			
NaN_3 (mM)			

[a]Maximal concentrations given in brackets.

Fractionation of cell-free rumen fluid. — A number of fractionation methods were investigated in attempts to purify the xylanase activity from cell-free rumen fluid. Most of them were aimed at removing materials of lower molecular weight that are present in rumen fluid, many of which absorb at 280 nm and give positive results in the protein determination, but some were also capable of fractionating polymers of higher molecular size. The methods used were: (*a*) ultra-filtration through an XM100A membrane (Amicon Ltd., High Wycombe). Other membrane filters (*e.g.*, XM50 and PM10) gave very similar results to the XM100A membrane; (*b*) molecular-sieve chromatography on various grades of Sephadex (Pharmacia Ltd., London); (*c*) fractional precipitation with ethanol; (*d*) fractional precipitation with ammonium sulphate; (*e*) chromatography on Dowex 1 (acetate form) ion-exchange resin. Attempts to fractionate the cell-free rumen fluid on columns of cellulose and Sephadex ion-exchange resins failed, as the protein(s) were adsorbed and could only

be removed with alkali. Treatment of the rumen fluid with charcoal and cellulose, as well as ether extraction, failed to remove completely the materials of lower molecular weight.

The fractions obtained were analysed for protein content (absorbance at 280 nm) and xylanase activity, and a typical set of results is shown in Table II. The xylanase activity is expressed as both per cent polysaccharide hydrolysed and per cent hydrolysed per unit of protein present. The results are also given in such form that the values are comparable. The bacterial pellet obtained from the same volume of rumen fluid was washed with artificial saliva and sonicated in an ice-bath so that the temperature did not rise above 10°. The volume of solution was adjusted with artificial saliva so that the final volume of sonicated material was the same as the volume of rumen liquor from which the bacterial pellet was obtained. In one experiment, the sonicated material was centrifuged at 50,000 *g* at 4° for 30 min to obtain a cell-contents fraction and a cell-wall fraction. The wall fraction was suspended in artificial saliva (the same volume as the cell contents), and the two fractions were incubated with a hemicellulose sample as described previously. A small amount of activity was detected in each fraction, but the overall amount was small compared to that found in the cell-free rumen fluid. From the percentage polysaccharide hydrolysed, the fraction retained by the membrane filter, the fraction of high molecular weight from the Sephadex column, two of the ammonium sulphate fractions, and one of the

TABLE II

HEMICELLULASE ACTIVITY OF FRACTIONATED CELL-FREE RUMEN FLUID[a]

Fractionation procedure	*Absorbance at 280 nm*	*Protein (μg/ml)*	*Polysaccharide hydrolysed* (%)	(% *per mg of protein*)
1 Cell-free rumen fluid	13.60	1735	21.4	12.3
2 XM100 ultrafiltrate	11.28	1400	4.5	3.2
3 XM100 concentrate	2.79	393	16.2	41.2
4 Sephadex G25 fraction of high molecular weight	6.62	615	18.2	29.6
5 Sephadex G25 fraction of low molecular weight	6.64	1159	6.9	6.0
6 Precipitate 50–67% EtOH	2.17	281	11.1	39.5
7 Precipitate 67–75% EtOH	1.10	145	317	25.5
8 Soluble in 75% EtOH	8.84	1305	0.8	0.6
9 Precipitate 20–30% $(NH_4)_2SO_4$ sat.	—	389	13.3	34.2
10 Precipitate 50–60% $(NH_4)_2SO_4$ sat.	—	275	16.4	59.6
11 Precipitate 70–80% $(NH_4)_2SO_4$ sat.	—	135	6.4	47.4
12 Dowex 1-X8 H_2O wash	4.66	486	15.4	31.7
13 Dowex 1-X8 30% HOAc wash	2.64	290	0.0	0.0
14 Sonicated bacterial pellet	1.06	276	5.2	18.8

[a]For equivalent volumes of rumen liquor.

ethanol fractions each had activity which was far greater than that of the sonicated bacterial pellet.

As a further guide to the composition of the fractions 1–14 in Table II, a sample from each of the fractions was chromatographed on a column of Corning CPG-10-175Å granules. The fourteen chromatograms are reproduced in Fig. 6. Chromatogram 1 shows that a large proportion of cell-free rumen fluid was material of low molecular weight which is positive to the Lowry protein assay. Protein components of high molecular weight were also present. Each of the fractionation methods examined gave some degree of separation, but the chromatograms of most importance were the ones from the samples which had high xylanase activity (3, 4, 6, 9, and 10). Samples 6, 9, and 10 all still contained material of low molecular weight. Only the material retained by the ultrafiltration membrane (3) and the component of high molecular weight from the Sephadex column (4) were devoid of this type of impurity.

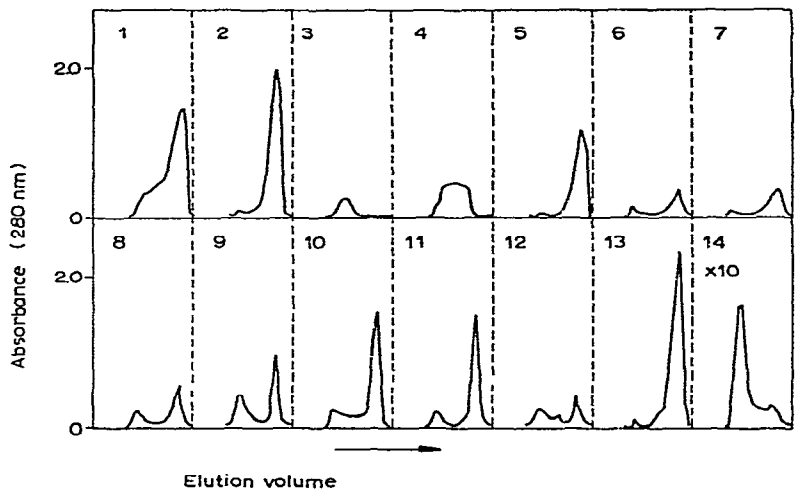

Fig. 6. Molecular-sieve chromatograms of partially purified rumen-liquor samples. For descriptions of the samples, see Table II.

DISCUSSION

It is possible that the hydrolysis of plant cell-wall carbohydrates by bacteria can occur at one of three sites: intracellularly, on the cell surface, or extracellularly. Although Howard *et al.*[4] and Beveridge and Richards[5] found that xylanase activity was present intracellularly, it seems unlikely to be the major site of polysaccharide degradation, since the d.p. of xylans reported in the literature is always estimated at greater than 50 and it would be virtually impossible for molecules of that size to be transported across the bacterial cell-wall. It is possible that hydrolysis could take place on the outer surface of the bacterial cell but, although xylanase activity was detected in both the cell-wall fraction and the cell-contents fraction obtained by

sonicating the bacterial pellet, the activities present in both of these fractions were low compared with that found in the cell-free rumen fluid and so it is believed that the major site of hemicellulose hydrolysis is extracellular. It must be emphasised here that it is not necessarily being suggested that the plant cell-walls are broken down to monosaccharides by extracellular enzymes. The hemicellulosic fractions (xylans) which we have used are isolated and soluble (or fairly soluble) substrates. Intact cell-walls containing these polysaccharides are rapidly degraded by whole rumen contents but the same cell walls are virtually unaffected by the action of cell-free rumen fluid. It may be that some preliminary hydrolysis, involving lignin–carbohydrate bonds, is required to release these polysaccharides from the cell wall.

The xylanase activity is not very stable, even when the cell-free rumen fluid is partially purified. This is not surprising, since cell-free rumen fluid contains a very complex mixture of proteins some of which will be from the feed and the rest derived from the bacterial and protozoal population. Amongst the latter group will be proteolytic enzymes[16]. Hence, the xylanase activity will be continuously destroyed. The diurnal variation in xylanase activity noted, especially when high levels of xylan were incubated, is probably caused by the soluble carbohydrates in the feed providing the energy sources for the microbial population during the immediate post-feed period. Only when that supply is used up (say 3 h after feeding) will the hemicellulase enzymes be produced to maintain the supply of fermentable carbohydrate to the microbial population.

The xylanase activity could be enhanced when reducing agents were added to the medium. All other types of reagents examined either had no effect or caused inhibition. The rumen contents *in situ* are highly reduced, so these conditions should be maintained *in vitro* for maximal activity. Since cell-free rumen fluid showed no hydrolytic or degradative activity towards a wide variety of monosaccharides, glycosides, and disaccharides, the substrates must be transported into the cell before being metabolised. There is evidence[19], however, that, as well as xylanase activity, cell-free rumen fluid contains hydrolytic activity towards a wide variety of polysaccharide substrates. To obtain maximal hydrolysis of xylans, there is an optimal range for the substrate-to-rumen liquor ratio. The optimal pH for hydrolysis was the rumen pH of 7.05, but the optimal temperature for hydrolysis was 31° and not the rumen temperature of 39°. The apparent "loss" in enzyme activity when incubations were carried out in the presence of cellulose was caused by irreversible adsorption of the xylan substrate onto the cellulose. This has been reported in other systems[17,18]. The rate and products of hydrolysis are being examined further, and the evidence to date suggests that relatively little monosaccharide is produced, the products being mainly oligosaccharides.

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