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# STUDIES ON THE HYDROLYSIS BY CARBOHYDRASES OF PLANT CELL-WALL CONSTITUENTS IN RELATION TO PASTURE QUALITY

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## SUMMARY

Enzyme preparations from rumen micro-organisms and the mould *Trichoderma viride* have been used to follow hydrolysis rates of herbage structural carbohydrates in neutral detergent residues from macerated plant tissues. Nylon bag digestion studies in the rumen on the same material suggest that the enzyme hydrolysis rates are relevant to rumen digestion. Differences have been detected in hydrolysis rates of cell walls from 'Grasslands Ruanui' and 'Grasslands Manawa' ryegrass with the Manawa material often, but not always, hydrolysed faster. Studies with isolated polysaccharides suggest that the differences may be due to either the hemicellulose A or the carbohydrates as an organized whole. Comparison of cell-wall preparations from oven- and freeze-dried material showed a slower enzymic hydrolysis and *in vivo* digestion for the oven-dried preparation from cocksfoot. The oven-dried ryegrass and clover preparations were hydrolysed a little slower than the freeze-dried ones by the rumen hemicellulase but no difference was found with the trichoderma enzyme or in *in vivo* digestion.

HERBAGE STRUCTURAL CARBOHYDRATES which, with lignin, form the fibre or roughage part of pasture are an important energy source for the ruminant. It has been suggested that differences in levels or organization of structural carbohydrates, particularly cellulose, may contribute to observed quality differences between ryegrass varieties (Bailey, 1964; Evans, 1964). Studies on digestion rates of these carbohydrates may, therefore, help in understanding the reasons for differences in pasture quality.

Plant structural carbohydrates may be briefly defined as fibres of organized, crystalline polysaccharide (cellulose possibly glucomannan) in an amorphous matrix of other polysaccharides (a complex of xylans, arabino-xylans and other polymers = hemicellulose). Lignin appears to be chemically linked to these matrix polymers. Plants are generally analysed for these polysaccharides by alkali fractionation (cellulose, insoluble) or acid hydrolysis (cellulose, most acid resistant) methods which are either tedious or imprecise. Thus the commonly used acid

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analyses for cellulose often include some xylan as cellulose. Studies on the ruminal digestion rates of these carbohydrates in feed have used either *in vivo* (e.g., Bailey, 1967) or *in vitro* (e.g., Tilley *et al.*, 1969) conditions with rumen micro-organisms. Such methods involve the analyses of undigested residues at each time interval and require relatively large samples. An early step in carbohydrate digestion in the rumen is hydrolysis to monosaccharides by microbial carbohydrases. The *in vitro* hydrolysis of plant structural carbohydrates in cell-wall preparations by these enzymes could provide a useful method of measuring hydrolysis rates which would be relevant to digestion conditions in the rumen. Such hydrolysis rates are moreover easily followed by simply removing small aliquots from the enzyme-substrate digest and measuring liberated monosaccharides by various colorimetric methods. Requirements are: (1) Active rumen microbial enzyme preparations which rapidly hydrolyse the polysaccharides to monosaccharides; and (2) Cell-wall preparations retaining some organization but free from soluble carbohydrates. Cell-free extracts from disrupted mixed rumen bacteria and protozoa rapidly hydrolyse plant hemicellulose fractions and  $\beta$ -glucan to monosaccharides (Bailey and MacRae, 1970) but these extracts have very poor cellulolytic activity. In studying the relationship between enzymically hydrolysed polysaccharide and digestibility, Jarrige *et al.* (1970) overcame this latter problem by using a fungal cellulase. We have followed the same approach by using the cell-free culture fluid of a strain of *Trichoderma viride* which contained powerful cellulase plus hemicellulase activity. Van Soest's (1966) neutral detergent process provides a residue from macerated plant tissue of cellulose + hemicellulose + lignin which still appears to retain the organization of the original tissue.

The present paper is an account of the use of these enzymes in studies on hydrolysis rates of herbage structural carbohydrates. *In vivo* digestion studies of the same herbage preparations using the nylon bag technique were also undertaken to evaluate the *in vitro* experiments in relation to rumen digestion.

## EXPERIMENTAL

### HERBAGE CELL-WALL PREPARATIONS

Bulk samples (500 to 1000 g) of leaves from single variety pastures or from single plants were cut about 7 to 10 cm above ground level and, where necessary, freed from any stems. Unless stated otherwise samples were

freeze-dried. Dried grass was either ground in a mill (1 mm) or macerated by chopping into 2 to 5 cm lengths, blending for 5 min at low speed in water (5 g in 250 ml) and finally filtering. Ground or macerated tissue was extracted with  $2 \times 250$  ml of boiling, neutral detergent (Van Soest, 1966) filtered and freeze-dried to give crude cell-wall preparations.

#### ENZYMES

Mixed rumen micro-organisms from hay fed sheep were disrupted in citrate-phosphate buffer (pH 6.5) and the centrifuged extracts dialysed against the same buffer and freeze-dried (Bailey and MacRae, 1970). The preparation hydrolysed plant hemicellulose (A and B),  $\beta$ -glucan and glucomannan completely but did not hydrolyse powdered cellulose. A local strain of *T. viride* was grown in cellulose-peptone media (Reese and Mandels, 1963) and the cell-free culture fluid dialysed against citrate-phosphate (pH 4.5) buffer and freeze-dried. This preparation completely hydrolysed cellulose,  $\beta$ -glucan and hemicellulose-A xylan but only partially (60%) hydrolysed hemicellulose-B.

#### DIGESTS

Conditions were such that the enzymes completely hydrolysed isolated polysaccharides in 24 hr. Digests containing freeze-dried enzyme (30 mg, rumen; 50 mg, trichoderma), cell-wall residue (10 to 15 mg containing 5 mg N-acid hemicellulose or 10 mg total polysaccharide) and water (10 ml, rumen; 5 ml, trichoderma containing 100  $\mu$ g/ml chloramphenicol) were incubated at 37° C with occasional shaking. At intervals 100 or 200  $\mu$ l portions were removed for analysis. Controls with substrate omitted were included in each run.

#### NYLON BAG DIGESTION

A modification of the technique described by Van Keuren and Heinmann (1962) was used in which small (20 cm  $\times$  5 cm) nylon bags (50  $\mu$  pore size) containing 1 g of cell-wall residue were attached to a heavy steel ring (300 g) and placed inside the rumen of a fistulated cow fed a hay diet. At intervals bags were removed, well washed in water by gentle squeezing and the contents removed and freeze-dried for analysis.

#### ANALYSES

Total reducing sugars in digests and acid hydrolyses were measured by the microcuprimetric method of Nelson

(1944) and glucose by glucose oxidase (Kilburn and Taylor, 1969). Polysaccharide contents of cell-wall preparations were measured by acid fractionation (Bailey, 1967) which divides them into N-acid hydrolysed (hemicellulose +  $\beta$ -glucan) and 72%  $H_2SO_4$  hydrolysed (cellulose + glucomannan + some xylan). Hydrolysis rates were expressed as liberated sugars in the digests calculated as a percentage of: (1) N-acid hydrolysed polysaccharide (rumen enzyme); and (2) Total (N-acid + 72% acid) polysaccharide (trichoderma enzyme). Lignin was measured by the acid detergent method of Van Soest (1963).

#### PLANT POLYSACCHARIDES

Hemicellulose-A and -B fractions and cellulose were prepared from grasses by alkali extraction (Gaillard and Bailey, 1968) after chlorite delignification.

#### RESULTS

##### STANDARD CONDITIONS FOR DIGESTION

Cell-wall preparations from single bulk samples of 'Grasslands Ruanui' and 'Grassland Manawa' ryegrasses (containing 10% hemicellulose, 16% cellulose, Ruanui; 11.5% hemicellulose, 15.0% cellulose, Manawa) were used in defining suitable enzyme-substrate concentrations for comparing grasses (Bailey and Jones, 1971). Results from a number of experiments showed that the conditions chosen gave nearly complete hydrolysis of hydrolysable polysaccharide in 24 hr incubation. Hydrolysis rates expressed as a percentage of substrate, were not affected by  $\pm 20\%$  variation in added substrate. Other comparisons showed no significant difference in hydrolysis rates between preparations from ground and macerated tissue with both enzymes. Delignification markedly increased the hydrolysis rates with the trichoderma enzyme but had little effect on the rates with the rumen hemicellulase. Cell-wall preparations from macerated, undelignified plant tissues were, however, chosen as the standard substrate in all subsequent experiments as being more comparable to the chewed plant tissue subjected to microbial attack in the rumen. Typical hydrolysis curves for both enzymes on macerated fibre preparations from the two grasses are shown in Fig. 1.

The rate of hydrolysis of Manawa was significantly greater than the rate of Ruanui ( $P < 0.05$ ). The same cell-wall preparations were digested in nylon bags in the rumen of a cow fed grass-clover hay. The results, shown in Fig.

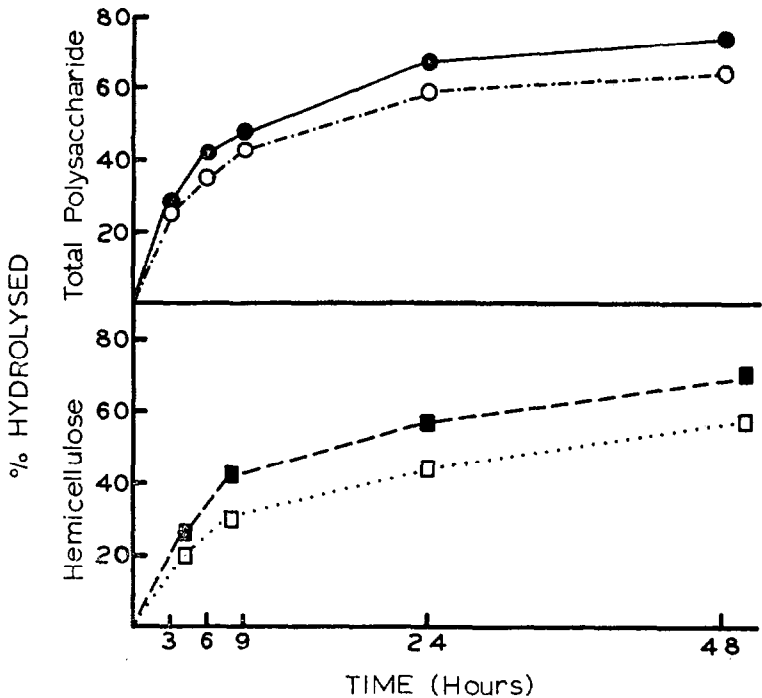


Fig. 1: Hydrolysis of ryegrass cell walls by carbohydrases.

Symbols — Manawa ryegrass: solid square, solid circle. Ruanui ryegrass: open square, open circle. Rumen enzyme: solid square, open square. Trichoderma enzyme: solid circle, open circle.

2, show an essentially similar rate of digestion to the trichoderma hydrolysis with the same difference between the two grasses.

Measurement of glucose in the trichoderma enzyme digests showed that 60 to 70% of the liberated sugar was glucose suggesting a greater rate of hydrolysis of cellulose than hemicellulose by this enzyme. However, some 5 to 10% of liberated sugar in the rumen hemicellulase digests was glucose, coming presumably from other  $\beta$ -glucan. The main problems of assessing rates of cellulose hydrolysis apart from the use of more specific enzymes are those of allowing for this  $\beta$ -glucan and of a suitable cellulose value (free from other glucan and xylan) as a basis for calculating results.

#### HYDROLYSIS OF ISOLATED POLYSACCHARIDES

An attempt was made to define the contribution of individual polysaccharides to the differences between the M

and R preparations as shown in Fig. 1 by measuring hydrolysis rates of various polysaccharides isolated from these cell walls. Cellulose preparations from both grasses were hydrolysed to completion by the trichoderma enzyme at the same rates. With hemicellulose preparations the rumen hemicellulase hydrolysed the more soluble hemicellulose-B preparations at the same rates but did hydrolyse Manawa hemicellulose-A (long chain xylan) about 10% faster than Ruanui hemicellulose-A.

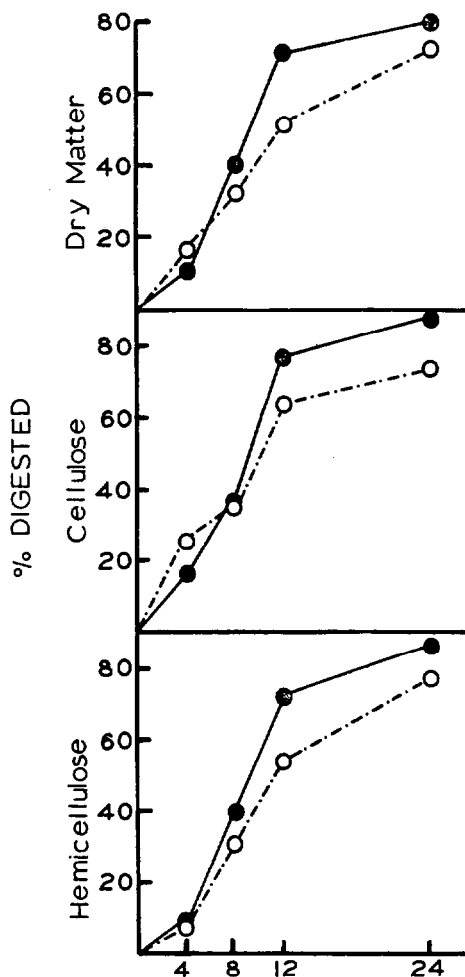


Fig. 2: Digestion in the rumen, in nylon bags, of ryegrass cell walls. Symbols — Manawa ryegrass: solid circle. Ruanui ryegrass: open circle.

While these results point to a contribution to the differences from xylan, this possibly is doubtful in view of the fact that this fraction forms only about 10% of the total structural polysaccharides. The possibility exists that the difference in rates between the two samples reflects an effect of the total polysaccharides as organized in the cell walls.

#### EFFECT OF HEAT DRYING OF HERBAGE ON HYDROLYSIS RATES OF CELL WALLS

The extent to which oven drying affects the digestibility and enzymic hydrolysis of cell-wall fibre has been examined by comparing enzymic hydrolysis rates and nylon bag digestion rates of cell wall preparations from duplicate samples of Ruanui ryegrass, cocksfoot and white clover which had been freeze-dried and oven-dried, respectively. As expected the oven-dried samples differed from the freeze-dried samples in having lower soluble carbohydrate and twice the apparent lignin content. Much of the protein in the oven-dried material being denatured now remained with the neutral detergent cell walls. Nevertheless, all of this protein was pepsin soluble and was digested from the nylon bag in the rumen within 24 hr. Table 1 summarizes the results for cocksfoot and shows some suppression in digestion and enzymic hydrolysis by oven drying. With the same preparations from both ryegrass and white clover, oven drying had no apparent effect on total dry

TABLE 1: ENZYMIC HYDROLYSIS AND DIGESTION OF CELL-WALLS FROM OVEN- AND FREEZE-DRIED COCKSFOOT

	<i>Cell-wall preparation from</i>			
	<i>freeze-dried*</i>		<i>oven-dried herbage*</i>	
	<i>% hydrolysed† or digested in (hr)</i>			
	24	48	24	48
(a) Enzymic hydrolysis				
Rumen enzyme	56.0	81.6	46.4	64.4
Trichoderma enzyme	53.9	75.6	42.4	58.4
(b) Nylon bag digestion				
Dry matter	82.1		77.8	
Hemicellulose (N-acid)	69.3		58.0	
Cellulose (72% acid)	72.2		57.4	

\*Composition of herbage, freeze-dried (oven-dried); hemicellulose 9.0 (10.0), cellulose 17.2 (16.3), protein 30.5 (30.6), soluble carbohydrate 8.8 (6.7), lignin 3.0 (4.9), neutral detergent residue = crude cell wall 31.6 (47.1).

†Calculated as % of hemicellulose (N-acid), total polysaccharide (N-acid + 72% acid) or cellulose (72% acid).



matter digestibility in the rumen or on trichoderma hydrolysis rates but slightly reduced the rate of hydrolysis by the rumen enzyme.

#### HYDROLYSIS OF RYEGRASS CELL WALLS FROM PASTURES AND SINGLE PLANTS

An important question with regard to herbage evaluation is the extent to which the difference in hydrolysis rates between the bulk samples of Ruanui and Manawa occurs consistently as such a difference could well help explain the postulate of Ulyatt (1970) that Manawa is broken down more quickly than Ruanui in the rumen. Samples of Manawa and Ruanui were available from two sampling periods of one of the grazing experiments described by Ulyatt (1971) during which sheep on Manawa were gaining weight faster than on Ruanui. Results with the trichoderma enzyme on the cell-wall preparations from these grass samples are given in Table 2 and show the same faster hydrolysis of Manawa as compared with Ruanui cell-wall preparations.

TABLE 2: ENZYMIC HYDROLYSIS OF RYEGRASS CELL-WALL PREPARATIONS

Ryegrass	Composition			Enzymic Hydrolysis			
	Neutral Detergent Residue (% of oven-dried weight)	Hemi-cellulose	Cellulose	Rumen Enzyme 24	Trichoderma Enzyme 48	(hr)	
						24	48
(a) Pasture (Ulyatt, 1971)							
Period 1							
Manawa	—	10.8	12.1			53	72
Ruanui	—	14.4	17.6			49	59
Period 2							
Manawa	—	11.8	10.6			55	68
Ruanui	—	12.4	14.3			50	57
(b) Single Plants (October 1970)							
Ariki	31.9	8.6	9.8	79	92	66	71
Ruanui	30.6	7.4	9.0	89	114	73	84
Manawa	29	7.4	9.9	83	100	66	72
N-Ireland	25.1	7.5	8.2	73	85	61	67
Spanish	27.5	7.7	9.4	76	89	66	69
S22	24.8	5.9	7.9	95	115	67	76
Tama	24.9	5.6	7.3	86	103	66	73
Paroa	27.4	7.3	8.8	76	89	63	70
LSD 5%	3.46	1.37	1.52	6.45	7.87	5.31	6.52
LSD 1%	5.11	2.03	2.24	9.52	11.62	7.84	9.62

During the 1970 season, samples of Manawa and Ruanui ryegrass were taken from areas of pure pastures under cages in which the grass was allowed to grow through to maturity. These results showed the expected more rapid hydrolysis of Manawa material during the earlier, leafy stage of growth; as the grasses mature this difference disappeared and the hydrolysis rate fell, for example, from 50 to 60% in 24 hr to 30 to 35% in 24 hr. A number of ryegrass varieties grown as single plants were also examined. The results from one cut showing a quite wide variation in hydrolysis rates of the cell walls are also listed in Table 2. These samples were taken from single plants growing in late spring to 35 to 40 cm high. The different growing conditions may be the reason for the faster hydrolysis rates of these cell-wall preparations compared with rates from pasture material. Samples from a later, early summer regrowth of these plants gave slower (50 to 65% hydrolysis rates. Grouping of the eight varieties in order of hydrolysis rates for these two cuts gave similar orders but consistency of the variations remains to be established.

#### DISCUSSION

It appears from the present studies that the use of carbohydrase preparations give measures of both the extent and rate of hydrolysis of herbaceous cell walls which are relevant to the *in vivo* rumen situation. Differences in rates may, therefore, be of some value in understanding pasture quality. Ulyatt (1970) has postulated a slower, more complete rumen digestion of Ruanui ryegrass compared with Manawa ryegrass as part of the explanation of the better weight gains of lambs grazing Manawa. The faster rate of hydrolysis of Manawa cell-wall preparations would fit this explanation. As Manawa often contains less structural carbohydrates than Ruanui this could further accelerate the effect of any difference in hydrolysis rates. It is interesting to note, however, that the difference in hydrolysis rates does not always occur, particularly in more mature grass.

It has been pointed out that various chemical analyses for cellulose, etc. have certain disadvantages. One of these is that the methods do not readily distinguish between lignified cell-wall carbohydrates which are largely unavailable to the rumen microflora and the digestible carbohydrates. As would be expected the trichoderma enzyme in particular does not hydrolyse all of the structural carbohydrates unless they are first delignified. Presumably the extent of enzymic hydrolysis of these carbohydrates in

undelignified tissue is a measure of the likely extent of interference by lignin in digestion. Similarly as the rumen hemicellulase preparation is unable to hydrolyse cellulose the extent of hydrolysis of hemicellulose by this enzyme in a preparation is a useful indication of the extent to which hemicellulose digestion requires prior removal of cellulose. In most cases quite a high proportion (50 to 65%) of hemicellulose is hydrolysable by this enzyme; the proportion can be much higher.

Several possibilities for further work are suggested from the data obtained in the present work. Apart from defining the contribution of cell-wall carbohydrates to pasture quality, it is possible that this approach points to a more meaningful method, so far as the ruminant is concerned, for measuring cell-wall carbohydrates in herbage. More specific enzymes which can be used separately or in conjunction with one another may give results more easily interpreted with regard to individual polysaccharides.

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