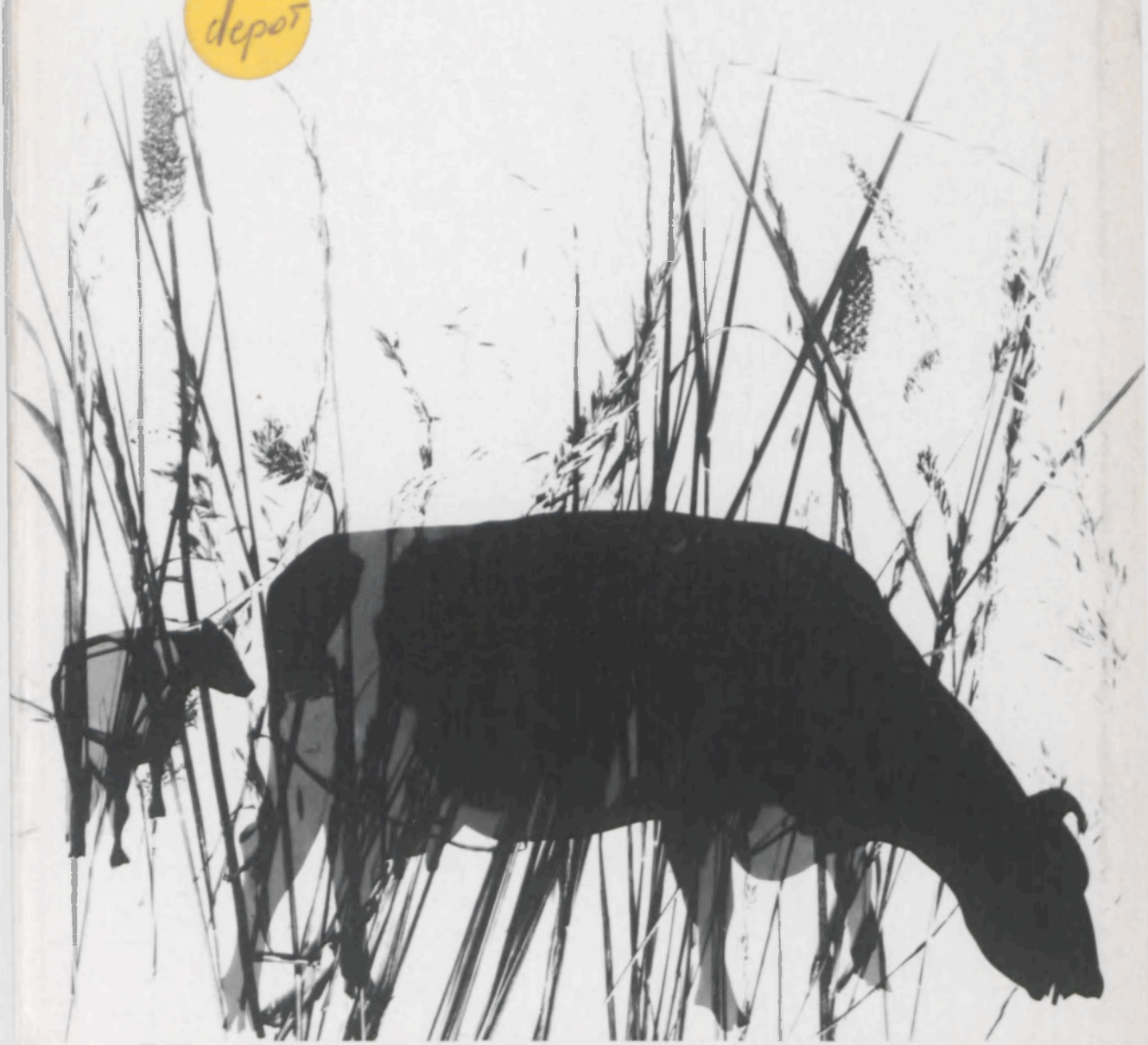
carbohydrate research in plants and animals



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LANDBOUWHOGESCHOOL WAGENINGEN, THE NETHERLANDS

CARBOHYDRATE RESEARCH IN PLANTS AND ANIMALS

Papers dedicated to Dr. Blanche Gaillard on the occasion of her retirement

H. VEENMAN & ZONEN B.V. – WAGENINGEN – 1976

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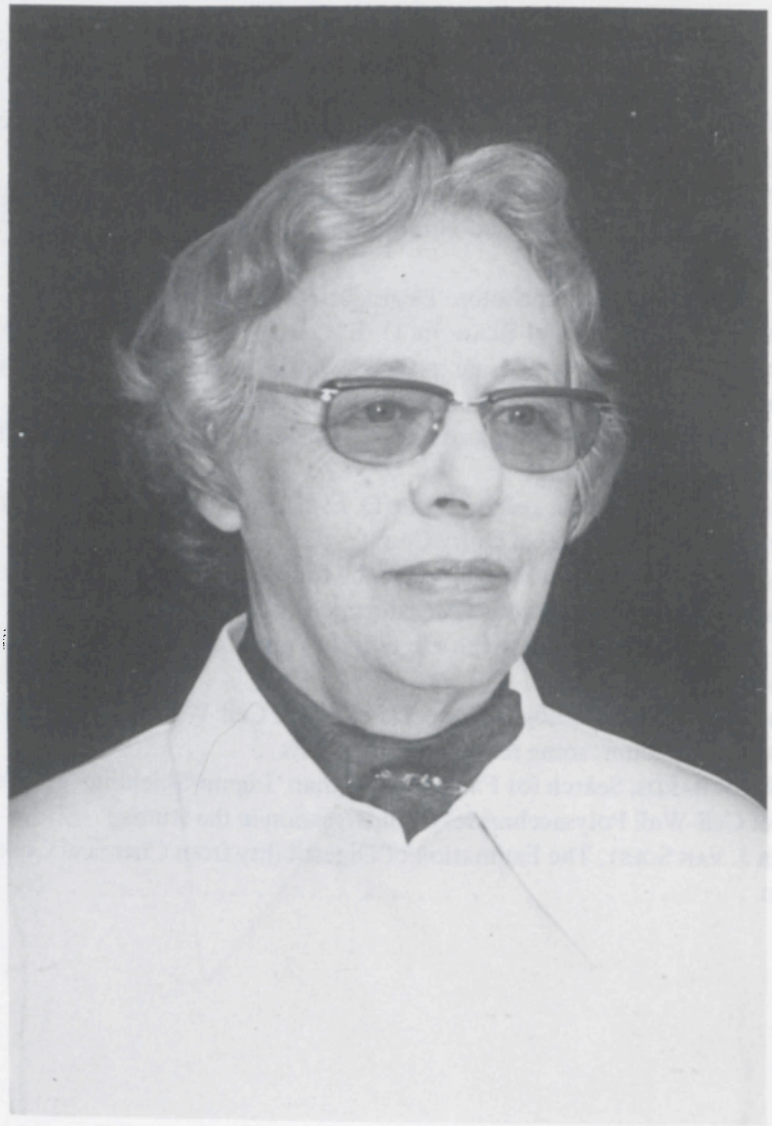

INTRODUCTION

On the occasion of the retirement of Dr. Blanche Gaillard from the Agricultural University at Wageningen we are publishing in her honour a special 'carbohydrates' issue.

In the field of research of carbohydrates from cell wall constituents Dr. Gaillard has worked intensively and has achieved international recognition through her publications.

To acknowledge her merits the following articles are written special for this issue by recognized research workers from various countries who readily dedicate the latest results of their carbohydrate research to Blanche Gaillard.

In this introduction I wish to outline briefly her biography to illustrate how education, broad experience and perseverance were the three pillars which supported her research talents.

After her grammar-school education and a one year stay in Switzerland, Dr. Gaillard studied pharmacy at the University of Utrecht where she qualified as a pharmaceutical chemist in 1937. After being assistant in the pharmaceutical laboratory of the University for a year, she managed independently a pharmacy in Nijmegen between 1938 and 1945. Next she entered the KNIL-army and departed as first lieutenant and military pharmacist to the former Netherlands Indies. As head of the chemical laboratory of the military hospital she was responsible for the organization of a dispensary in which she had to identify and test for quality drugs left by the Japanese occupying forces. She was also appointed as lecturer in pharmaceutics at the University of Batavia.

After returning in 1948 to the Netherlands she went again to Indonesia in 1949 and until 1951 held the position of head of the chemical laboratory of the criminological institute. In 1951 she joined the staff of the Laboratory of Animal Physiology of the Agricultural University at Wageningen, retiring in 1975.

Initially, Prof. Dr. E. Brouwer offered her a 3-year contract during which time she had to study the cell wall constituents of roughages. This study resulted in her thesis entitled: 'Chromatographic characterisation of the polysaccharides of the cell wall with regard to the analysis of roughages'.

As a result of this achievement the Agricultural University decided to enlist her in permanent service. In doing so a broad research field was opened for Blanche Gaillard which she has since explored intensively, as is apparent from her numerous publications. She has paid much attention to the methods of analysis of structurally different carbohydrates present in various feed plants and in different growth stages. Her investigations on the interaction of polysaccharides with iodine are well known and were carried out with the objective of devising a method for the separation of linear from branched polysaccharides. Apart from her research work in plants on the identification of carbohydrates, she has exerted her strength to determine the digestibility

of various carbohydrate fractions in ruminants and to relate the chemical composition of the roughages with the feeding value.

Blanche has carried out many investigations in cooperation with other research workers and in various non-European laboratories she was invited to study special carbohydrate problems of mutual interest. As a guest worker she joined research teams in the United States, Australia and New Zealand where she contributed to local carbohydrate research work. On account of her knowledge, experience and ability to communicate she has become a source of information for national and international colleagues. The Agricultural University at Wageningen and especially the Laboratory of Animal Physiology are very grateful for the research performed by Blanche.

By publishing this special 'Gaillard' issue they, together with the authors, express their gratitude.

P. W. M. van Adrichem

HERBAGE HEMICELLULOSE AND ITS DIGESTION BY THE RUMINANT

R. W. BAILEY, J. A. MONRO, S. E. PICKMERE and A. CHESSON

Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand

SUMMARY

Herbage hemicellulose is described in terms of its chemical composition, association with other plant constituents and interlinkage in plant cell walls.Results on the digestion of herbage hemicellulose in the ruminant from studies of rumen micro-organisms and their enzymes and also from *in vivo* studies are reviewed. Recent work on the use of model enzyme systems with total herbage cell wall preparations and on hemicellulose digestion in the post ruminal digestive tract are also reviewed. The need to look at hemicellulose digestion in total plant cell wall tissues is stressed. Lignin is generally assumed to be the main inhibitor of plant hemicellulose digestion in the ruminant. There is a need to investigate the effect of other possible inhibitors, particularly O-acetyl groups.

INTRODUCTION

The non-cellulosic structural polysaccharides of herbage represent a major source of ruminant energy. These compounds, commonly grouped under the terms pectin and hemicllulose are, together with cellulose, lignin and some glycoprotein the interlinked organised system which forms the plant cell walls. Likewise their digestion in the ruminant is part of a process involving the utilization of this plant cell wall as a whole entity. This digestion involves initially enzymic hydrolysis by various rumen microbial carbohydrases to release sugars which can then be fermented to volatile fatty acids in the usual manner. It is at this initial stage that differences in the microbial attack on the various plant cell wall polysaccharides are largely evident and when such properties as degree of lignification affect the extent of enzymic hydrolysis.

Chemical methods can effectively separate plant cell walls into more or less distinct chemical entities such as xylans (hemicellulose), β-glucan, cellulose and pectin although there may be some cleavage of covalent links involved in the separation. Most earlier work on hemicellulose digestion in the ruminant has been concerned with the enzymic hydrolysis of such chemically isolated polymers by extracts from rumen bacteria and protozoa. This work includes that of BLANCHE GAILLARD with extracts from mixed rumen bacteria and rumen protozoa mentioned below. While these *in vitro*, enzyme studies give an indication of the first stage of hemicellulose digestion they only

explain part of this initial process as the hemicellulose is presented to the rumen microflora in organised plant cell walls not as isolated polymers. For this reason detailed studies must involve the use of total plant cell wall preparations. Further as some plant tissue escapes from the rumen undigested or is indigestible in the rumen the possibility of hemicellulose digestion in the lower digestive tract must be considered.

The main purpose of this paper is to acknowledge the contribution BLANCHE GAILLARD has made to understanding the digestion of herbage roughage in the ruminant, with particular reference to hemicellulose, by briefly reviewing some recent studies in this field. The present paper does not attempt to review all research on herbage hemicellulose or its digestion in the ruminant. Rather it will be concerned with some recent aspects of (a) current attempts to define chemically the organised cell wall structure of herbage, (b) ruminal breakdown of hemicellulose, (c) the use of mould carbohydrases as an *in vitro* model for studying the rumen enzymic hydrolysis of hemicellulose in intact plant cell walls and (d) hemicellulose digestion in the post ruminal tract. Recent research at Applied Biochemistry Division in these areas is in part an extension of hemicellulose work done by Dr. GAILLARD during her two most welcome and fruitful visits to this Division.

HERBAGE HEMICELLULOSE

Chemical components in isolated hemicellulose

Plant hemicellulose is usually considered chemically in terms of the polymers extracted from delignified plant cell walls by dilute and strong alkali and separated by subsequent fractionation. Delignification is commonly applied because it is a necessary pre-treatment for total (from hardwoods) or any (from softwoods) extraction of hemicellulose from these woody tissues. A detailed account of the main hemicellulose polymers prepared from the two main classes of herbage, grasses and legumes is given by BAILEY (1973). They belong to two main groups; (a) *pentosans*, which are principally xylans based on chains of β1–4 linked xylose with various amounts of arabinose, uronic acid and methyl uronic acid substituents and (b) *hexosans*, which are based either on chains of β1–4 linked glucose and mannose (glucomannans and galactoglucomannans) or on chains of β1–3 β1–4 linked glucose (β-glucans). Another type of xylan, previously reported in seeds (KOOIMAN, 1960) called xyloglucan and consisting of β1–4 linked xylose chains with β linked glucose side chains has recently been detected in sycamore suspension cultures (ASPINALL *et al.*, 1969) and postulated to be present in many plant cell walls (KEEGSTRA *et al.*, 1973). Although the same types of hemicellulose polysaccharides appear common to most angiosperms there are species differences particularly between grasses and legumes. Grasses tend to have more hemicellulose than legumes, with more of it xylan and with some of their xylans differing from legumes in the extent of arabinose and uronic acid substitution (e.g. WHISTLER and GAILLARD, 1961; GAILLARD, 1965). Polysaccharides based on glucomannan appear to be absent from grasses but are present in small amounts in legumes (GAILLARD

and BAILEY, 1968). While xyloglucan may be present in many plant tissues it appears to be absent from grasses (BURKE *et al.*, 1974). Alkali soluble polymer glucose extrated from grasses is often present as pentose-free β1–3 β1–4 linked glucan; these polysaccharides have been isolated by fractionation of hemicellulose from several temperate grasses (FRASER and WILKIE, 1971; BUCHALA and MEIER, 1973).

Compounds associated with hemicellulose in plant tissue

Closely associated with plant cell wall hemicellulose is the pectic material. In herbage this usually consists of main chains of rhamno-polygalacturonic acid with arabinogalactan side chains. Legume herbage typically contains much more pectin than grasses. Another polymer closely associated with hemicelluose and now known to be present in small amounts in the primary cell walls of many plants is the glycoprotein term 'extensin' by LAMPORT (1970). This consists of a peptide rich in hydroxyproline with arabinose oligosaccharides and sometimes galactoaraban side chains. The other major polymer closely associated with hemicellulose is lignin. There is now much evidence that lignin is covalently linked to the non-cellulosic cell wall polysaccharides (HARKIN, 1973) and such has been found to be the case in grasses (MORRISON, 1974). Grass cell walls have also been shown to contain phenolic monomer-carbohydrate constituents which can be released by enzyme action (HARTLEY, 1973). O-Acetyl groups are another hemicellulose substituent which may affect solubility and inhibit enzyme action. Most of the xylose units in hardwood xylans carry O-acetyl groups (BOUVENG, 1961) and this has now been shown to be the case in grass xylan where up to half of the xylose groups may be O-acetylated (BACON *et al.*, 1975). All of these constituents; pectin, 'extensin', lignin monomers and O-acetyl together with cellulose must be allowed for in understanding both the nature of hemicellulose in the intact plant cell walls and its ruminant digestion in these whole walls. The fact that some of them, for example O-acetyl groups, are completely lost from the hemicellulose during conventional alkaline extraction needs to be considered when working with such extracted fractions.

Extraction of hemicellulose

Alkali remains the most useful solvent for the direct extraction of plant hemicellulose with subsequent separation into hemicellulose A (xylan precipitated by acid at 0°C) and hemicellulose B (more complex polymers not acid precipitated). For accounts of the fractionation of hemicellulose see BLAKE and RICHARDS (1971) and BAILEY and CONNOR (1972). Solvents such as guanidine thiocyanate and 8 M urea which only cleave hydrogen bonds can dissolve some but not all hemicellulose from primary cell walls (MONRO *et al.*, 1975b). A useful empirical measurement of cell wall hemicellulose uses the detergent extraction techniques of VAN SOEST which involve extraction of plant tissue first with neutral detergent (VAN SOEST, 1965) to dissolve solubles, protein and pectin followed by acid detergent (VAN SOEST, 1963) to remove hemicellulose and leave ligno-cellulose. This sequential process not only gives a mea-

sure of hemicellulose but also provides depectinated but largely intact cell wall material for enzyme digestion studies. With legumes, but not so much with grasses, it is important that the processes be used sequentially to remove pectin in the neutral detergent as direct use of acid detergent does not dissolve this pectin (BAILEY and ULYATT, 1970).

With lignin-free primary plant cell walls the delignification of conventional alkali extraction is unnecessary; all of the hemicellulose is directly soluble in alkali (MONRO *et al.*, 1974). Such alkali extraction can give a significant hemicellulose separation if it is done sequentially at 0 °C and 18–20 °C. Most of the hemicellulose, including all of the hemicellulose A xylan, is extracted at 0 °C while arabino-galacto-glycoprotein and complex hemicellulose B material is extracted at the higher temperature (MONRO *et al.*, 1975a). Pectic polysaccharide is not removed by the alkali at either temperature (BAILEY and KAUSS, 1974) although it is dissolved in the much milder ammonium oxalate at higher temperatures. When alkali extraction is applied to leafy grass tissue very low in lignin (3 %) most of the hemicellulose is extracted; with grass higher in lignin (9 %) less polysaccharide is dissolved (BAILEY and PICKMERE, 1975). Such extractions could be due to the hemicellulose in immature grass being either only hydrogen bonded to cellulose, unattached to lignin but acetylated or only associated with lignin by alkali-labile links. It seems that more attention could well be paid to the extent of direct alkali solubility of herbage hemicellulose in relation to potential digestibility. In mature roughage lignification would largely inhibit alkali extraction of hemicellulose although even here alkali treatment does render such straw or hardwood hemicellulose more digestible in the rumen (CHANDRA and JACKSON, 1971).

Hemicellulose in plant cell walls

For many years the accepted model for plant cell wall organisation involved a network of crystalline cellulose fibres embedded in a matrix of amorphous hemicellulose and lignin with pectic material largely in the middle lamella as an inter-cellular cement. Apart from possible lignin-carbohydrate links covalent links between the polymers were not postulated. Evidence is now accumulating to show that the cell wall is more highly organised with both covalent links and non-covalent links such as hydrogen bonds between the various carbohydrate polymers. In fact Albersheim and co-workers (KEEGSTRA *et al.*, 1973) have proposed a cell wall structure in which all of the polymers are linked into one large molecule. In this structure both glycoprotein (via galactan) and xyloglucan (via pectic arabino-galactan) are linked covalently to rhamno-polygalacturonic acid (pectic material) and this complex is hydrogen bonded through the xyloglucan to cellulose. This structure takes no account of arabinoxylan or β-glucan and may not apply at least to grass cell walls. Likewise the solubility of much primary wall hemicellulose in guanidine thiocyanate and the differential alkali solubility of hemicellulose and glycoprotein at 0° and 18–20 °C coupled with alkali insolubility of pectin do not fit the proposed structure. It seems likely therefore that in many plant cell walls much of the hemicellulose is either not

covalently bound to the other wall constituents or only through very alkali labile links. Non-covalently bound hemicellulose must be at least hydrogen bonded otherwise it would be water soluble rather than need guanidine thiocyanate for extraction. A modified wall structure proposed by MONRO, BAILEY and PENNY (unpublished) envisages much of the hemicellulose and all of the pectin unliked to other constituents and only some of the hemicellulose bonded to the glycoprotein and cellulose. As mentioned other evidence points to the covalent linkage of lignin into such cell walls during secondary thickening and particularly to the hemicellulose by alkali labile links. It is also evident that cellulose is not the only possible crystalline polymer in plant cell walls as hemicellulose xylan has been shown capable of forming microcrystallites within the matrix (NEIDUSZYNSKI and MARCHESSAULT, 1971).

These studies on cell wall organisation further stress the need to be clearly aware of the simplifications in plant cell wall polysaccharide structure and enzymic digestion that are inherent in work with polymer fractions isolated by chemical means. In addition to inhibition of enzyme attack by substituents such as lignin and acetyl groups cross linking of polysaccharides will lead to inhibition of enzymic degradation unless all of the required carbohydrases are present. Likewise in highly ordered hydrogen bonded systems such as micro-crystalline xylan enzyme accessibility and action may be completely inhibited.

HEMICELLULOSE DEGRADATION IN THE RUMEN

Rumen micro-organisms and their hemicellulases

Rumen bacteria have long been known to degrade plant hemicellulose and ferment the released sugars (see e.g. HUNGATE, 1966). The associated rumen protozoa have also more recently been shown to be capable of using plant hemicellulose (e.g. BAILEY *et al.*, 1962). As with the other major natural, aerobic classes of digesters of plant fibre, soil bacteria and saprophytic fungi, the initial step in rumen hemicellulose digestion is enzymic hydrolysis to simple sugars by a range of carbohydrases such as xylanases. Unlike the saprophytic fungi however the rumen organisms do not usually secrete extracellular hemicellulases or cellulase; rather the enzymes remain closely associated with the cells. The hemicellulase complex can however be readily extracted from both rumen bacteria and protozoa by simple cell disruption; in contrast the cellulases can not be so easily extracted. Although mould and soil bacteria hemicellulases (endo-xylanases; random cleavage) and cellulases have been investigated in great detail with extensive fractionation studies, possibly because the enzymes are readily obtained from cell-free culture fluids, studies on rumen microbial hemicellulases have been much more limited. The common substrate used to isolate hemicellulose fermenters from the rumen is purified xylan and many authors have shown that extracts of mixed rumen bacteria (e.g. WALKER and HOPGOOD, 1961) or specific rumen xylan fermenters (e.g. HOWARD *et al.*, 1960) can hydrolyse this type of xylan. Both GAILLARD *et al.*,

(1965) and BEVERIDGE and RICHARDS (1973a) have shown that extracts of mixed rumen bacteria can hydrolyse all of the hemicellulose fractions of herbage. While GAILLARD *et al.*, (1965) found differences in the rates of hydrolysis of the various fractions by the enzymes, BEVERIDGE and RICHARDS (1973a) found no difference. Major studies involving the fractionation of hemicellulases from specific rumen organisms have been those of HOWARD *et al.*, 1960 on the endo-xylanases from *Butyrivibrio fibrisolvens* and other rumen bacteria and the detailed fractionation of the hemicellulose complex from the protozoa *Epidinium ecaudatum* by BAILEY and GAILLARD (1965). In this latter case an endo-xylanase, a xylodextrinase, an arabinofurorosidase and a β-glucanase were separated. In a different approach hemicellulose fractions other than xylan were used to isolate rumen bacteria; although strains of the same species usually isolated on xylan (*B. fibrisolvens*) were obtained (CLARKE *et al.*, 1969) the strains did show differences between one another in the hemicellulase activities of their enzyme extracts.

Studies on the actual carbohydrases in hemicellulases from extracts of isolated rumen micro-organisms using defined, isolated substrates give us only a limited idea of the patterns of hemicellulose digestion in the rumen where the mixed population are hydrolysing intact herbage particles. For this reason recent interests have been more directed towards studies of digestion either by cultures of specific rumen bacteria growing preferably on total plant hemicellulases or by *in vivo* studies of hemicellulose digestion in the rumen itself.

DEHORITY has made a study of the degradation of various herbage hemicelluloses by rumen bacteria including both cellulolytic bacteria and strains isolated on xylan. From his findings he postulated (DEHORITY, 1967) that cellulolytic bacteria were as important as other rumen bacteria in hydrolysing herbage hemicellulose and found differences in the utilization of isolated hemicellulases from different herbages by the same bacteria. Some cellulolytic strains were shown to degrade hemicellulose but were unable to utilize the degradation products (DEHORITY, 1968). When these organisms were supplied with intact forage instead of isolated hemicellulose (COEN and DEHORITY, 1970) similar results were obtained. The hemicellulose-utilizing strains attacked the herbage hemicellulose in the forage to different extents which also varied for different herbage species at various growth stages. The non-hemicellulose-utilizing cellulolytic strains were also able to degrade the hemicellulose in the forage. There was also evidence for synergysm, particularly between the hemicellulose utilizers and non-utilizers.

Hemicellulose digestion in ingested forage

In vivo digestion studies where total hemicellulose or hemicellulose fractions in feed and faeces are measured give other indications of the nature or hemicellulose breakdown in the ruminant, but not necessarily in the rumen only. Such studies with temperate herbage and roughages show that maturity and lignification affect the digestion of hemicellulose, that hemicellulose is less digestible than cellulose and that hemicel-

lulose xylan is less digested than hemicellulose arabinose (GAILLARD, 1962; JARRIGE and MINSON, 1964; JONES, 1970; FORD, 1973). In similar digestion studies using several temperate and tropical grasses and differential detergent analyses, MCLEOD and MINSON (1974) suggested that herbage cell wall carbohydrates including the hemicellulose should be divided into a lignin-free fraction which is totally digested and a lignified fraction which is undigested. A similar division for cellulose digestion has been proposed by WALDO and co-workers (SMITH *et al.*, 1971). This effect of lignin is further supported by the finding by GAILLARD and RICHARDS (1975) of soluble lignin-carbohydrate compounds in rumen fluid and presumably released by enzymic attack on plant cell walls. These compounds apparently survive in the rumen and pass out undigested. Studies on speargrass hemicellulose (BEVERIDGE and RICHARDS, 1973b) also indicate that lignification could be an inhibitor of the digestion of some herbage hemicellulose in the rumen.

Few results have been reported on the actual rate of digestion, as opposed to extent of digestion, of herbage hemicellulose in the rumen. BAILEY (1967) followed the loss of total fractions including hemicellulose and cellulose in the rumen of cattle by emptying the rumen at intervals, mixing and sampling. Results with fresh clover or clover hay showed a steady loss of ingested hemicellulose during 24 hours digestion; the rate was generally similar to the rate of loss of cellulose. Monosaccharide analyses of the hemicellulose indicated that arabinose was removed at a faster rate than xylose. In a different approach (DEKKER *et al.*, 1972; DEKKER and RICHARDS, 1973) the rate of loss of hemicellulose from tropical grass and legume herbage was followed in nylon bags suspended in the rumen. Hemicellulose was digested slowly and incompletely with more extensive loss of arabinose compared to xylose. Hemicellulose fractions extracted after delignification of the undigested residue from these experiments was readily hydrolysed by rumen hemicellulases (BEVERIDGE and RICHARDS, 1973b) which was considered to indicate the protective effect of lignin.

FORD (1973) compared the composition of individual hemicellulose fractions from feed and faeces and found from the faecal results preferential removal of hemicellulose arabinose, glucose and galactose relative to xylose, together with a proportional increase in hemicellulose-A xylan in the faeces to the feed. We have also examined the hemicellulose composition of ryegrass and the faeces from a sheep fed on the same grass. The results showed that all grass hemicellulose fractions were present in the faeces with a slightly lower proportion of hemicellulose B relative to other hemicellulose. Compared to feed fractions arabinose was lower in faecal hemicellulose B but not in the other fractions.

Hemicellulose in rumen and faecal particulate matter

In considering much of the work discussed in this section emphasis has been laid on the need to work with intact digesting plant particles. Such work is further complicated by the physical properties (see VAN SOEST, 1975) and size variation of these particles which are also changing during digestion. Particle size affects not only digestion

Table 1. Hemicellulose in ryegrass and faeces.
A. Total fractions

Hemicellulose fractions	Feed	Faeces
	(% of dry weight)	
10% Alkali soluble hemicellulose A	8.44	5.98
10% Alkali soluble hemicellulose B	13.51	9.42
24% Alkali soluble hemicellulose	5.52	6.11
Cellulose + lignin	23.40	22.50

B. Monosaccharide composition

	Xylose	Arabinose	Glucose	Galactose
	(all relative to xylose)			
Hemicellulose A, feed	1.0	0.84	–	–
Hemicellulose A, faeces	1.0	0.21	–	0.01
Hemicellulose B, feed	1.0	0.43	0.07	0.03
Hemicellulose B, faeces	1.0	0.10	0.01	–
24% Alkali hemicellulose, feed	1.0	0.26	0.03	–
24% Alkali hemicellulose faeces	1.0	0.30	0.12	–

but also escape from the rumen possibly before digestion is complete. The distribution of hemicellulose fractions and cellulose in particulate matter of different sizes from various parts of the ruminant digestive tract do not appear to have been examined. Such studies are complicated by the presence of rumen bacteria in the smaller particle fractions. In a preliminary study we have examined the distribution of hemicellulose xylose and cellulose glucose in various size particles from rumen contents, after 24 hours starvation, and faeces from a cow fed grass hay. The results, Tables 2

Table 2. Particle size distribution in rumen contents and faeces*.

Fraction number	Passing sieve size	Rumen contents	Faeces
		(% of total dry weight)	
1	2.0 mm	17.0	11.4
2	1.0 mm	16.7	17.1
3	500 μ	15.4	15.2
4	250 μ	10.8	16.3
5	125 μ	8.7	6.5
6	filtrate (centrifuged)	31.5	43.4

* From fistulated cow fed grass hay, starved overnight before rumen sampling.

Table 3. Hemicellulose xylose and cellulose glucose in rumen and faecal particulate matter.

Sample number*	Rumen Contents		Faeces	
	Hemicellulose xylose	Cellulose glucose	Hemicellulose xylose	Cellulose glucose
	(% of dry weight of each total fraction)			
1	15.3	26.8	9.0	22.1
2	9.3	22.2	7.3	16.7
3	7.0	24.6	6.9	17.8
4	8.3	22.3	7.3	14.4
5	4.4	9.9	9.6	11.2
6	4.2	9.2	4.8	9.8

* See Table 2.

and 3, indicate a clear difference between the large and small particles. A more detailed analysis of the hemicellulose of the particles did not show any other differences between the particle fractions from the rumen or faeces. Compared to the feed all of the rumen and faeces samples showed less hemicellulose B relative to hemicellulose A, while faeces hemicellulose B but not hemicellulose A had less arabinose than the feed samples. These latter results are in agreement with those already reported.

On the whole all of the results from ruminant studies stress the need to study rumen hemicellulose digestion in total plant cell walls in the presence of cellulose digestion: that is *in vivo* or in a model system where both classes of enzymes are exerting maximum effect. While lignification is often postulated as the limiting factor in hemicellulose digestion, there is a need to be able to distinguish between hemicellulose unavailability because of cell wall structural organisation of carbohydrates *per se*, interference by cellulose, lignification or other factors such as acetylation.

In Vitro Hemicellulose Digestion

In vitro laboratory studies of herbage digestion have been developed largely in order to predict *in vivo* digestibility and voluntary intake and to help define herbage quality. As the well known *in vitro* methods using mixed rumen bacteria are almost always used to measure total digestible material in herbage samples they tell us little about the digestion of individual plant polymers in the herbage. A more recent *in vitro* technique which can give more specific indications of the likely digestion of fractions such as hemicellulose uses depectinated cell walls and mixtures of mould cellulase plus hemicellulase. Highly active enzyme mixtures are readily obtained form cell-free culture fluids of *Trichoderma viride* (Bailey and Jones, 1971) and other moulds (Hartley *et al.*, 1973; Jones and Heywood, 1975). Under suitable conditions the rate and extent

of hydrolysis of cell wall hemicellulose and cellulose is closely analogous to the hydrolysis of the same material in the rumen (BAILEY and JONES, 1971) so that the model appears to be valid. The method can be used as a simple *in vitro* one to measure potential digestibility of herbage cell walls (GUGGHOLZ *et al.*, 1971; JONES and BAILEY, 1974; JONES, 1975; MCQUEEN and VAN SOEST, 1973). Correlations of digestibility or voluntary intake with enzymically hydrolysable total cell walls are as good, if not better than, those from *in vitro* digestibility (JONES and BAILEY, 1974; JONES, 1975; JONES and HEYWOOD, 1973, 1975). With more specific analysis of the cell wall preparations the method can be used to study various aspects of enzymic hydrolysis of specific polymers such as hemicellulose in total plant cell walls (e.g. BAILEY and JONES, 1971, 1973; HARTLEY *et al.*, 1973). An important aspect of the approach is that it permits a distinction to be made between hemicellulose and cellulose which is accessible to enzyme attack and that which is not so accessible; a distinction which cannot be made by chemical analysis. Rumen microbial hemicellulase, which is easily prepared free from cellulase, can also be used comparatively in the same system to obtain some indication of the extent to which hemicellulose hydrolysis is dependent on concurrent cellulose hydrolysis (BAILEY and JONES, 1971, 1973).

Pectic substances interfere with the enzymic hydrolysis (JONES and BAILEY, 1974) and must be first removed with neutral detergent. In grasses very low in lignin delignification is also still necessary to achieve maximum mould enzymic hydrolysis (BAILEY and JONES, 1971). The fact that this delignification does not increase the hydrolysis of the residual hemicellulose by rumen hemicellulase suggests that this residual hemicellulose is intimately associated with cellulose as well as lignin.

When the mould system was applied to cell walls from leaves of several ryegrass varieties at a similar stage of growth (BAILEY and JONES, 1971, 1973) marked differences between the varieties in extents of hemicellulose hydrolysis were obtained. Within each variety the extent of hydrolysis fell with increasing maturity. The technique has also been used to examine the effect of oven-drying on herbage hemicellulose and cellulose digestion (JONES and BAILEY, 1972), being particularly useful here as the denatured protein and Maillard reaction products (VAN SOEST, 1975) produced during drying interfere with the usual detergent analyses. Results with both ryegrass and clover showed that quite prolonged oven-drying with the production of large amounts of denatured protein did not affect the extent or rate of enzymic hydrolysis of the cell wall hemicellulose and cellulose; this was in agreement with *in vivo* digestion of the same material. Either as a simplified technique or as a more complex one based on comprehensive analysis of the cell walls the use of mould carbohydrases offers a useful approach for investigating the possible ruminal mode of enzyme attack on constituents of intact, organised plant cell walls.

Hemicellulose Digestion in the Post Ruminal Digestive Tract

The bulk of herbage hemicellulose and cellulose digestion occurs in the rumen. However as much undigested hemicellulose and cellulose passes down the digestive tract to be voided in the faeces there is the possibility of digestion in this lower tract, particularly as Bailey and McCrae (1970) were able to extract hemicellulase activity from sheep caecal bacteria. Quantitative results from the uncannulated animals used in this work did not however indicate much post ruminal digestion of hemicellulose in the roughage fed. The development and wider use of the technique of cannulation of the post ruminal tract has enable digesta passing down the tract to be examined both quantitatively and qualitatively. In initial studies using this technique with hay-fed animals Gaillard and Van 't Klooster (1969) and MaCrae and Armstrong (1969) concluded that hemicellulose digestion was largely confined to the rumen except when large amounts of starch were included in the diets (MaCrae and Armstrong, 1969). More extensive studies using cannulated sheep on a variety of herbage and roughage diets (Thomson *et al.*, 1972; Beever *et al.*, 1971; Beever *et al.*, 1972; Ulyatt and MaCrae, 1974; Egan *et al.*, 1975) indicate however that some 10–30% of herbage hemicellulose is digested in the large intestine. These results also show consistently that the percent of digested hemicellulose digested in the large intestine is always greater than the percent of digested cellulose digested in the same region (Ulyatt *et al.*, 1975). An analysis of these and other unpublished results (Ulyatt, pers. commun.) show that in herbage and roughage as the hemicellulose digestibility increases the percent digested in the rumen increases. The effect of added cereal starch recorded by MaCrae and Armstrong could be a special case. Differences between the results where no post ruminal hemicellulose digestion was detected and those where it did occur may be explained in terms of feeding technique, feed used or flow measurement methods. It is perhaps relevant that in the enzyme studies of Bailey and MaCrae (1970) both rumen and caecal hemicellulases possessed only limited hydrolytic activity against hemicellulose in particulate material leaving the rumen of the sheep used. The particulate fractions (Table 2) isolated from well digested rumen contents were likewise not hydrolysed by a mould hemicellulase and pectinase from *Rigidoporus lineatus* and only slightly hydrolysed (1%) by hemicellulase and cellulase from *T. viride*. Detailed enzymic and microbial studies of hemicellulose, and cellulose, degradation by post ruminal digestive tract bacteria from sheep fed on diets with high post ruminal hemicellulose digestion would seem to be worthwhile.

Conclusion

A fairly clear picture of the nature of herbage hemicellulose digestion in the ruminant has emerged in the past 10–15 years. This digestive process is more complex than initial rumen studies using isolated hemicellulose fractions seemed to indicate. More

meaningful results come from studies using intact plant cell wall materials. Much plant hemicellulose remains undigested by the ruminant and while it is customary to ascribe this non-digestion to lignification, other factors such as xylan crystallinity, hemicellulose-cellulose interaction and O-acetyl groups may be responsible and need investigating. It is also evident with herbage-fed animals that post ruminal digestion of hemicellulose is important and warrants further investigation. Going beyond the ruminant, faecal hemicellulose which is resistant to rumen or fungal hemicellulase attack is presumably degraded in the soil or digested in faecal digestion systems such as methane generators. If this latter case is true then the microbiology of plant hemicellulose digestion in anaerobically fermenting rumenant faeces seems worth exploring.

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BIOCHEMISTRY OF 5-THIO-D-GLUCOSE*

MINSHEN CHEN and ROY L. WHISTLER

Department of Biochemistry
Purdue University West Lafayette, Indiana 47907

INTRODUCTION

D-Glucose is the central carbohydrate metabolite of almost all living things. It is commonly called blood sugar, corn sugar or even corn syrup and, as a hydrolysis product of cereal starch, provides more than 50% of the caloric intake of the human race. Its nearest analog, both chemically and physically is 5-thio-D-glucose (figure 1)

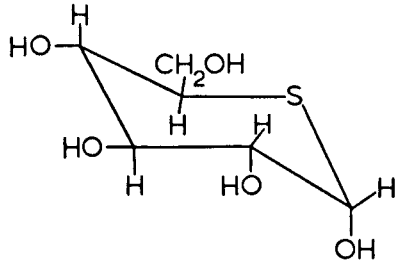

Figure 1. Structure of 5-thio-D-glucose

in which a sulfur atom replaces the normal ring oxygen atom. While 5-thio-D-glucose has many chemical properties similar to real D-glucose, the sulfur sugar is quite different biochemically and it has recently shown interesting properties as a temporary male antifertility agent and even as an antiparasitic agent. One of its derivatives, namely the analog of uridine diphosphoglucose, has shown remarkable stimulating action on the enzyme glycogen synthetase.

SYNTHESIS AND CHEMICAL PROPERTIES

It was some time ago that our laboratory undertook the first synthesis of 5-thio-D-glucose (FEATHER and WHISTLER, 1962). The process was rather long as shown in the

* This is a report.

equations (figure 2) and the yield depended heavily upon the skill of the operator. Consequently, the process was improved by NAYAK and WHISTLER (1969) to give a yield of 33% starting with D-glucose and subsequently several shorter synthetic routes were developed (CHIU and WHISTLER, 1973; EL-RAHMAN and WHISTLER, 1973). It was interesting that the sugar analog crystallized in the α-D-anomeric form and was very stable, exhibiting little mutarotation in solution and no evidence of oxidation to disulfide. Apparently the greater thermodynamic stability of the thiol hemiacetal does not allow significant ring opening, producing the stability observed and a lack of mutarotation. Nuclear magnetic resonance indicated that the ring form had the stable Cl or $^4C_1$ conformation common to D-glucose and depicted in the above structure (SUZUKI and WHISTLER, 1972).

Although little disulfide formation occurs on air oxidation, it is possible to oxidize the sugar acetate with periodate to the sulfoxide level whereupon a new chiral center

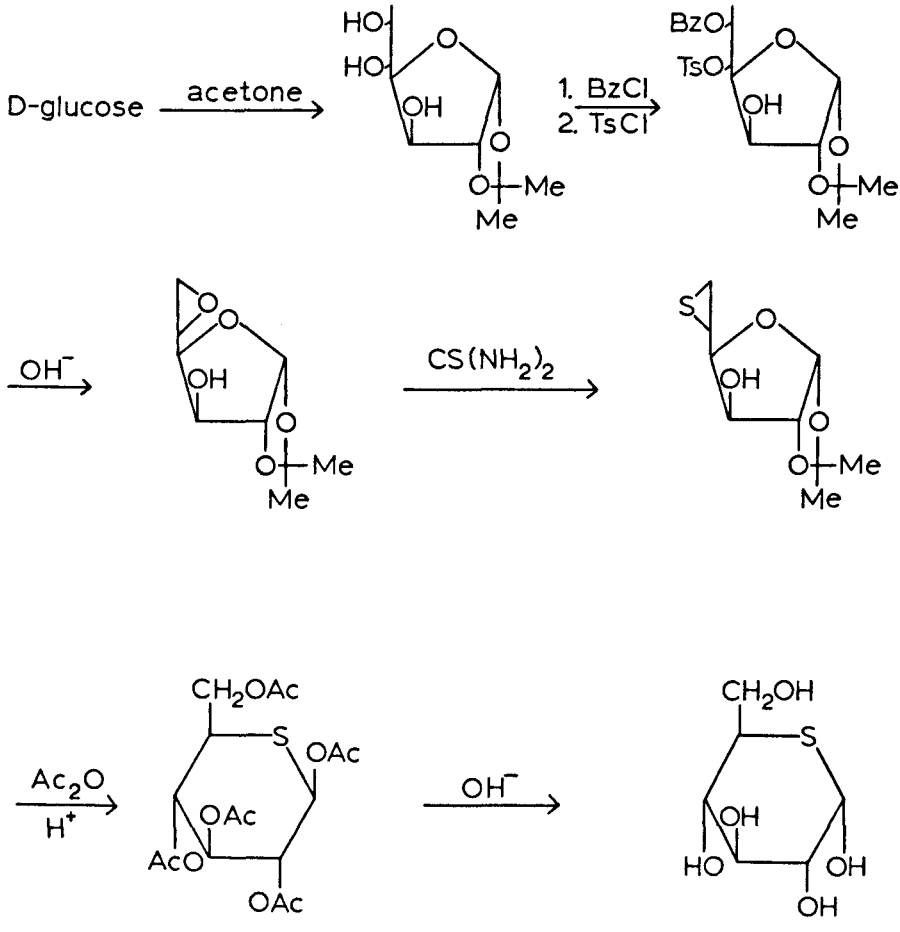

Figure 2. The synthesis of 5-thio-D-glucose

is produced and two separate diastereomers are obtained. At this point, every atom in the ring is optically active. Further, stronger oxidation produces the sulfone of natural lower stability (ROWELL and WHISTLER, 1966).

Glycosides of sulfur sugars are more easily acid hydrolyzed than normal sugar glycosides possibly because of the greater stability of the sulfonium-carbonium transition state over that of the oxonium-carbonium transition state (WHISTLER and VAN ES, 1963) (figure 3).

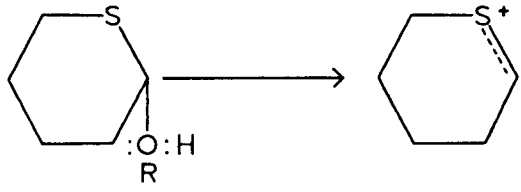

Figure 3.

INHIBITION OF MEMBRANE TRANSPORT

When 5-thio-D-glucose was first made it was added to cultures of *Lactobacillus casei* and *Saccharomyces cerevisiae* and found not to support growth (FEATHER, 1963). Since only small quantities of sugar analog were initially available it was next examined by feeding to *Drosophila melanogaster* and *Periplaneta americana*, the American cockroach (SHANKLAND *et al.*, 1968). At low concentration in the fruit fly diet, 5-thio-D-glucose interfered with utilization of D-glucose in the development of larvae. Diets containing a 5-thio-D-glucose to D-glucose molar ratio of 0.03 resulted in only 45% normal development of larvae into the pupal stage. Sugars in the molar ratio of 0.31 prevented all development of larvae into the pupal stage. This inhibition of fruit fly development was postulated as a consequence of 5-thio-D-glucose inhibition of D-glucose transport across cell membranes and/or its interference with one or more enzymes involved in carbohydrate metabolism. The experiment suggested that the sugar analog was absorbed and distributed in the fruit fly body. Therefore, the absorption and distribution of the sulfur sugar was examined in the adult cockroach. The sugar analog was observed to be readily absorbed from the gut and distributed in the haemolymph, fat body and the three portions of the gut in essentially equal proportions as is D-glucose. In insects, the haemolymph and midgut are sites for synthesis and breakdown of glycogen and trehalose and contain a number of intermediates and enzymes for glycolysis and the Krebs cycle (CHEFURKA, 1965; KILBY, 1963).

5-Thio-D-glucose was next examined by feeding to rats (HOFFMAN and WHISTLER, 1968). When given orally it was found to be acutely nontoxic showing an LD_{50} of 14 g/kg. When large single doses (50–100 mg/kg) of thiosugar were intraperitoneally administered to rats a rapid rise in blood sugar level occurred reaching a maximum in about 2.5 hours and returning to normal in about 6 hours. In nearly the same time

period glucosuria was present from renal spill-over from the high blood sugar levels. This condition too, returned to normal in about 6 hours. Booster injections every hour could maintain the hyperglycemic and glucosuric conditions. However, administration of insulin completely nullified the hyperglycemic effect. An interesting observation noted that 97.3% of a single administration of 5-thio-D-glucose was recoverable as unchanged thiosugar from the excreted urine within a 24 hour period although most of the analog was excreted within the first 6 hours of peritoneal injection. This rapid excretion of unchanged sugar analog was interesting in view of the strong physiological effect it produced and its rapid excretion indicated the short duration observed for the hyperglycemia. Fasted rats given the sugar analog in a single dose of 50 mg/kg lost 50% of their liver glycogen within a 2.5 hour period. A half hour after a 150 mg/kg dose of thiosugar a 73% increase in nonesterified fatty acid was present in the blood and urinary catecholamine increased 43%. These observations were considered as suggesting that the sugar analog inhibited cellular uptake of D-glucose and led to increased alternative metabolism of fats.

The suggestion that 5-thio-D-glucose inhibits transport of D-glucose across cell membranes and thereby decreases carbohydrate metabolism was borne out by examination of the transport inhibition of the sugar analog in tissue sections of rat liver, kidney and diaphragm (WHISTLER and LAKE, 1972). In initial work kidney and diaphragm tissue uptake of D-glucose was reduced by 35% and 76% respectively, while in liver there was a net D-glucose output at all dose levels probably due to glycogen breakdown. Inhibition of thiosugar analog to other transport had already been observed. BARNETT *et al.* (1970) and CRITCHLEY *et al.* (1970) examined the effect in hamster small intestine and found inhibition to the accumulation of actively transported D-galactose. KABACK (1968) observed that the thiosugar inhibited transport of methyl α-D-glucopyranoside in a cell transport preparation of *Escherichia coli* by a noncompetetive inhibition of phosphoenolpyruvate-phosphotransferase system. The sulfur sugar appears to be a less effective stimulator of receptor sites in *Sarcophaga bullata* (JAKINOVICH *et al.*, 1971) than is D-glucose. CRITCHLEY *et al.* (1970) also observed inhibition to methyl α-D-glucopyranoside transport in kidney cortex. Somewhat later 5-thio-D-glucose was observed to inhibit insulin release by the pancreatic beta cells (HELLMAN *et al.*, 1973). In fact, a 10 mM concentration of thiosugar completely inhibited insulin release. This inhibition of insulin release may result from an inhibition of D-glucose transport across the beta cell membrane restricting its triggering of insulin secretion.

Quantitative measurements on the inhibition of 5-thio-D-glucose to membrane transport of D-galactose and methyl α-D-glucopyranoside in kidney cortex slices and diaphragm slices showed that with D-galactose in kidney the inhibition constant, K_i, to be 4.85 mM (WHISTLER and LAKE, 1972). The analog inhibited facilitated diffusion of D-xylose in rat diaphragm muscle but had no effect on passive diffusion of D-arabinose.

5-Thio-D-glucose affects other processes in addition to sugar transport. For example, it inhibits active transport in rat kidney cortex of neutral amino acids, such as

glycine, cycloleucine and aminoisobutyric acid (WHISTLER and LAKE, 1972). KAHLENBERG *et al.* (1972) demonstrated that the sulfur sugar did not bind to human erythrocyte membranes with the same affinity as D-glucose, suggesting that the sulfur in the pyranose ring reduces to the normal D-glucose receptor site.

5-Thio-D-glucose is itself actively transported by the D-glucose transport system (WHISTLER and LAKE, 1972). Both in kidney cortex (WHISTLER and LAKE, 1972) and in hamster intestine (CRITCHLEY *et al.*, 1970) the sulfur sugar is transported against a concentration gradient and here, as in kidney transport, is Na^+ dependent and phlorizin sensitive. A Lineweaver-Burk plot of accumulation of 5-thio-D-glucose in kidney cells yields a K_m of 2.4 mM and a V_{max} of 70 μmole/h per gram of cell water.

ANTIFERTILITY ACTION

Having established that 5-thio-D-glucose inhibits D-glucose transport attention was turned to potentially useful affects which might be produced or controlled by the thiosugar. Two tissue effects immediately come to mind that might be advantageously brought under control by alterations in transport of D-glucose. One is the inhibition of D-glucose transport on spermatogenesis and another is the effect of such transport inhibition on intracellular parasites or parasites with high D-glucose requirements.

There is ample evidence that spermatogenesis is particularly sensitive to D-glucose concentration. DAVIS (1969) shows that addition of 9 mM of D-glucose to incubated sections of rat testis greatly increases amino acid incorporation into protein. Other work emphasizes the importance of D-glucose for maintaining normal morphological appearance and function of the rat testis. Reproductive function in the human male is markedly affected by the presence of hyperglycaemia. Diabetic men have increased incidence of impotence (SCHOFFLING, 1963), a decreased sperm count (BABBOTT *et al.*, 1958), poor sperm motility (KLEBANOW and MACCLEOD, 1960) and atropic changes in the germinal epithelium of the testes (BABBOTT, 1958). A mass of data implies that D-glucose is a significant factor in testicular function.

To examine the effect of 5-thio-D-glucose on sperm production, the sugar was mixed with mouse ration and fed at different daily dose levels to mice (ZYSK *et al.*, 1975). At daily dose levels of 30 mg/kg and above sperm development was completely inhibited within a two week period and animal infertility continued for as long as the sugar analog was given, although the animals' libidos were not effected. During the treatment period the blood sugar level of the mice remained normal. Histological observation of testes of animals given 5-thio-D-glucose showed effects, the onset of which depended on the dose level. The initial histological change is seen within a week for mice ingesting 100 mg/kg and within two weeks for mice ingesting 20 mg/kg. Initial change is the occurrence of a few enlarged spermatogenic cells in the lumen of the seminiferous tubules or the spermatid zone of the germinal epithelium. After two weeks on the higher doses or three to four weeks on the low dose regine, the number of

hypertrophied cells increased and the greatest numbers were formed within the lumina of tubules that retained the earlier cell stages of spermatogenesis but few or no spermatids. At this stage of alteration the hypertrophied cells became degenerate, as indicated by the formation of cytoplasmic vacuoles and irregular contour of the nucleus. Spermatogenesis was still active in most tubules and spermatozoa were present in epididymal tubules.

As the testicular changes became more severe, there was a marked reduction in the number of spermatogenic cells and an absence of spermatids and spermatozoa from the tubules of the epididymis.

Giant cells appeared in a number of seminiferous tubules. These cells were extremely large with abundant darkly eosinophilic cytoplasm and nymerous nuclei arranged either in a wreath-like fashion at the periphery of the cytoplasm or as centrally or eccentrically located masses. Some of these cells had undergone necrosis as the nuclei were either pyknotic or had undergone karyorrhexis accompanied by cytolysis. As more and more of the syncytial giant cells became necrotic, tubules becoming filled with eosinophilic masses and necrotic giant cells. Some tubules were atrophic and filled with calcified debris.

The most severely affected testes were small and contained only atrophied tubules that had few or no germinal cells or a few syncytial giant cells but were lined by viable Sertoli cells.

Normal sperm development was seen in mice five to six weeks after treatment (30 mg/kg) was discontinued but eight weeks were required to bring to normal weight and appearance the testes of those animals given higher dose levels (100 mg/kg) of thiosugar. Sperm regeneration at eight weeks was observed after all dose levels. Litters sired by the animals tested were normal in size and the general health of both F_1 and F_2 progeny was healthy.

These preliminary results establish that 5-thio-D-glucose can completely inhibit spermatogenesis and fertility in male mice and that removal of the analog from the diet allows full recovery of spermatogenesis and fertility.

BUSHWAY (1975) demonstrates that incorporation of ^{14}C-uridine into testicular ribonucleic acid was inhibited more than 50% in rats fed daily doses (50 mg/kg) of 5-thio-D-glucose for two weeks. After five weeks, incorporation of ^{14}C-lysine was reduced to 60%. In rat testicular slices, 5-thio-D-glucose, unlike D-glucose, did not increase ^{14}C-lysine transport.

ANTIPARASITIC ACTION

The tapeworm *Hymenolepis diminuta* like other tapeworms and many other parasitic cestodes has a high D-glucose requirement (READ and SIMMONS, 1963). The sugar is actively transported by an energy requiring, Na^+ dependent system that is sensitive to inhibition by various glycosides and sugar derivatives (DIKE and READ, 1971; READ *et*

al., 1974; PAPPAS and FREEMAN, 1975). Transport of D-glucose is thus similar to that in mammalian intestinal epithelium and kidney tubules (GUYTON, 1971).

Administration of 5-thio-D-glucose by way of drinking water to rats harboring *H. diminuta* inhibits growth of the parasites compared to growth of the parasites in control rats (K. R. KAZACOS unpublished data). After 11 days at dose levels of 150 mg/kg and 200 mg/kg of body weight worms removed from rats given 5-thio-D-glucose showed an average of 76.5% and 71.2% reduction in dry weight respectively compared to those from control rats. In some instances fewer worms were present. This occurred even though the blood sugar level of the rats remained normal.

EFFECT ON TUMOR CELLS

Since the early observation by WARBURG (1930) that some tumor cells are more glycolytic than normal cells, especially under aerobic conditions, their carbohydrate metabolism has interested investigators. The high rate of glycolysis exhibited by some tumor cells may be a consequence of increased capacity for transport of substrate (EAGLE *et al.*, 1958; RENNER *et al.*, 1972; HATANAKA *et al.*, 1969; MARTIN *et al.*, 1971; SINGH *et al.*, (1974), increase in activity of glycolytic enzymes, phosphofructokinase, hexokinase, pyruvate kinase (SINGH *et al.*, 1974; RUBENCHIK, 1974) or changes in the feedback mechanisms of hexokinase or phosphofructokinase (RUBENCHIK, 1974). The predominance of glycolysis in cancer cells eliminates the functioning of the opposing pathway, gluconeogenesis, increases the potential for the production of D-ribose and NADPH and results through isozyme shifts in cancer cells in a decreased susceptibility to regulatory signals that arise from other cells (WEBER, 1974). The similarity of tumor metabolism in cells arising from different causes suggests a dependence on D-glucose.

The effect of 5-thio-D-glucose on cultured tumor cells was initially investigated by BUSHWAY (1975). It was found that the sugar analog at a concentration of 6.3×10^{-4}M substantially reduced cellular protein accumulation in nasal pharyngeal KB, hamster brain tumor cells (HBT) and mouse less Remia (L1210) cells, while a concentration of 2.6×10^{-3}M was required for 50% reduction in cellular protein for cervical carcinoma (HeLa) cells. The sugar analog at a concentration of 6.3×10^{-4}M also effectively reduced cell numbers of KB, HBT and L1210 while a concentration of 1.3×10^{-3}M was required to reduce the numbers of HeLa cells. It is not established whether the action is due to inhibition of D-glucose transport or to inhibition in the glycolytic pathway although CHEN and WHISTLER (1975a) have found that 5-thio-D-glucose somewhat inhibits phosphorylation of D-glucose by hexokinase and that phosphates of the sulfur sugar strongly inhibit phosphoglucomutase catalysis of D-glucose-1-phosphate conversion to 6-phosphate.

Absence of Effect on Learning and Memory of Rats

D-Glucose is necessary for the maintenance of brain tissue function (Himrich *et al.*, 1939; Olsen *et al.*, 1950). Because of the inhibitory effect of 5-thio-D-glucose on D-glucose transport its effect on the blood brain barrier and consequently on the brain is of great significance. Quantitative results on any effect on the blood brain barrier are still not complete but behavioral experiments with rats show no untoward effect on the animals brain. Thus, Bushway (1975) demonstrate that 5-thio-D-glucose on feeding to rats at 100 mg/kg/day had no effect on the ability of animals to learn maze problems nor to remember solutions to problems. Intelligence was measured using Hebb-Williams maze problems. Although 5-thio-D-glucose may effect centers of the brain other than those tested, careful examination of animals receiving the sugar analog have not revealed abnormal behavior.

Metabolism of 5-Thio-D-glucose

Oral administration or intravenous injection of radioactive thiosugar to rats confirms the rapid elimination from the animal system of approximately 95 % of the sugar, table 1 (Pitts *et al.*, 1975). A small amount is oxidized to carbon dioxide. The low rate of metabolism agrees with the low phosphorylation coefficient of 6.3×10^{-4} with hexokinase (Chen and Whistler, 1975a).

To examine the thiosugar phosphates they were made both chemically and biochemically.

Table 1. Distribution of radioactivity after intravenous and oral administration of 5-Thio-D-[U-^{14}C]glucopyranose*.

Component analyzed	5-Thio-D-[U-^{14}C]glucopyranose	
	iv** (%)	oral (%)
CO^2 6 h	1.0 ± 0.3	0.8 ± 0.1
12 h	–	0.1 ± 0.01
24 h	–	0.05 ± 0.01
48 h	–	0.08 ± 0.03
72 h	–	< 0.01
Total	1.0 ± 0.3	1.1 ± 0.2
Urine	93.0 ± 3.1	53.5 ± 3.6
Feces	–	36.7 ± 2.0
Total excreted	93.0 ± 3.1	90.2 ± 6.0
Carcass	1.6 ± 0.2	4.2 ± 2.5
Intestine	–	0.2 ± 0.07
Glycogen	0.5 ± 0.3	0.08 ± 0.07

* Results are reported as the numerical average of the percent of the administered dose for three rats ± average deviation from the numerical average.

** iv – intravenous administration.

5-Thio-D-glucose-1-phosphate was prepared by acetylating the thiosugar to the 1, 2, 3, 4, 6-penta-O-acetyl derivative, treatment of this with 30% hydrogen bromide to prepare 2, 3, 4, 6-tetra-O-acetyl-5-thio-D-glucopyranosyl bromide which was reacted with silver diphenylphosphate to give the 1-phosphate derivative. Blocking diphenyl groups were removed by hydrogenation and remaining acetyl groups by ester interchange in methanol with lithium hydroxide catalysis. The thiosugar 1-phosphate was obtained in 59% yield (WHISTLER and STARK, 1970).

5-Thio-D-glucose-6-phosphate was made from the methyl 5-thio-α-D-glucopyranoside by diphenylphosphorochloridation and removal of phenyl groups by hydrogenation (WHISTLER and STARK, 1970). Thiosugar phosphate can also be synthesized by phosphorylation of 5-thio-D-glucose with ATP at pH 8 (CHEN and WHISTLER, 1975a).

Chemical synthesis of uridine-(5′-thio-α-D-glucopyranosyl pyrophosphate) (GRAHAM, 1974) proceeded through the condensation of uridine 5′-monophosphate 4-morpholine-N, N′-dicyclohexylcarboxamidine salt with 5-thio-D-glucose-1-phosphate. This UDGP analog termed UDPTG could also be made biochemically with standard enzymes by usual methods.

ENZYMATIC BEHAVIOR OF 5-THIO-D-GLUCOSE AND IIS DERIVATES

5-Thio-D-glucose serves as a substrate for yeast hexokinase. The analog competitively inhibited D-glucose phosphorylation. 5-Thio-D-glucose-1-phosphate is a substrate for phosphoglucomutase. Transfer of phosphate from 1-phosphate to 6-phosphate of 5-thio-D-glucose follows the normal kinetic mechanism of D-glucose-1-phosphate. 5-Thio-D-glucose-1-phosphate is a potent inhibitor of D-glucose-1-phosphate conversion to 6-phosphate (CHEN and WHISTLER, 1975a).

The product, 5-thio-D-glucose-6-phosphate produced from either thiosugar phosphorylation or conversion from the 1-phosphate, can serve as a substrate with glucose-6-phosphate dehydrogenase. Thus, 5-thio-D-glucose metabolism might follow the pentose phosphate shunt. 5-Thio-D-glucose-6-phosphate is competitive to D-glucose-6-phosphate and noncompetitive to NAD^+ (M. CHEN and R. L. WHISTLER, unpublished data).

Although 5-thio-D-glucose-1-phosphate does not replace D-glucose-1-phosphate as a substrate, 5-thio-D-glucose-1-phosphate is a potent competitive inhibitor toward D-glucose-1-phosphate and a noncompetitive inhibitor toward amylopectin or glucogen for phosphorylases. 5-Thio-D-glucose competitively inhibits amylopectin action with potato phosphorylase and noncompetitively inhibits D-glucose-1-phosphate and phosphate. 5-Thio-D-glucose acts as competitive inhibitor to D-glucose-1-phosphate for glycogen phosphorylase a and b and noncompetitively inhibit glycogen and phosphate. Thus, 5-thio-D-glucose and its 1-phosphate could interfere with the supply of D-glucose from polysaccharide degradation (CHEN and WHISTLER, 1975b, c).

UDGP is of central importance in the interconversion of sugars and oligo- and

polysaccharides. UDPTG is similar in physical and chemical properties to the natural nucleotide derivate. However, it is very unusual in some of its biochemical properties. For example, UDPTG appears to be a potent activator of rat liver glycogen synthetase *a* even though it is not a substrate. At 1.0 mM UDPTG causes over 400% activation of glycogen synthetase *a* activity. Activation by UDPTG is accompanied by normalization of the otherwise sigmoidal kinetics for UDPG with glycogen synthetase *a* and a decrease in the apparent K_m for UDPG from approximately 2.0mM to 0.62 mM. UDPTG inhibits catalytic at higher concentrations. UDPTG has no apparent effect on glycogen synthetase *b* activity.

The surprising activation of activity by UDPTG at low concentrations, followed by an inhibitor of activity at higher concentrations suggest that the UDPTG can bind initially to a few UDPG sites causing stimulation of binding to unoccupied sites. At higher concentrations UDPTG begins to saturate the UDPG sites leading to inhibition of catalysis. The fact that UDPTG binds to effector and/or active sites but is not converted to product gives it particular usefulness for examining enzyme action as well as substrate interaction and allosteric effects (T. GRAHAM and R. L. WHISTLER, unpublished data).

CONCLUSIONS

It is evident that 5-thio-D-glucose is an interesting biochemical analog of D-glucose. While early indications suggest the strong possibility of it becoming useful as a non-toxic, non-hormonal agent for control of male fertility, it also shows promise as a means for examining in greater detail various aspects of enzyme activity.

Furthermore, while the effect of 5-thio-D-glucose to inhibit spermatogenesis and even its action as an antiparasitic agent may first appear due only to inhibition of membranal transport of real D-glucose the principal effect or at least a secondary effect may be at the metabolic enzyme level through possible action of the thiosugar or its derivatives on carbohydrate metabolizing enzymes. Detailed examination of effects on regulatory enzymes needs further investigation. Replacement of the ring oxygen of a sugar by the next periodic table family element sulfur can cause a significant and interesting change in the resultant sugar.

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EFFECT OF AGE, LEAF NUMBER AND TEMPERATURE ON CELL WALL AND DIGESTIBILITY OF MAIZE

B. DEINUM*

Department of Field Crops and Grassland Husbandry, Agricultural University, Wageningen, The Netherlands

SUMMARY

In an indoor trial effect of temperature and age on growth and development of individual leaves of maize was investigated.

The first developed leaves of maize were highly digestible and low in cell-wall constituents (% cwc), whereas later leaves were less digestible and richer in % cwc, presumably because of a larger contribution of the highly lignified, poorly digestible midrib.

A higher temperature caused a small increase in cwc, but a sharp drop in digestibility, which could be attributed to the extremely large effect of temperature on cell-wall digestibility of the midrib. Digestibility dropped somewhat with age, at low temperature less than at high temperature.

These results were related to the lignification of the various tissues. It was therefore recommended to breed for forages less subject to lignification and with a smaller contribution of lignified tissues like vascular bundles and sclerenchyma.

INTRODUCTION

The age of herbage is an important criterion for quality, and numerous estimates have been made of the effect of age on forage quality under the prevailing regional conditions. However, there have been only a few investigations into the more fundamental aspects of this age effect.

Usually the total herbage has been collected and a conglomerate of green and dead leaves, sheaths and stems has been investigated, each tissue having its own pattern of deterioration of quality with ageing. Therefore to collect more information on the effect of age. DEINUM and DIRVEN (1971) investigated separate leaves of a maize crop, and found that digestibility dropped somewhat with age but also that later developed leaves were much less digestible than the early developed leaves at the same stage of development. Similar effects of leaf succession and age were found in Australia with *Panicum maximum* (WILSON 1973a), in Nigeria with *Andropogon gayanus* (HAGGAR and AHMED, 1971) and in Puerto Rico with *Digitaria decumbens* (VAN SOEST, 1972,

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personal communication), all experiments being conducted in the field under prevailing environmental conditions. DEINUM and DIRVEN (1971) suggested that the effect of leaf succession in the maize crop was caused by the lower temperature in spring during development of the earliest leaves and the higher temperatures when the later leaves were developing. So the question arose whether the lower digestibility of later developed leaves were caused by a higher temperature during development or by some intrinsic factor. The trial here presented attempted to answer this question.

MATERIAL AND METHODS

In October 1971, 10 seeds of maize cv Pioneer 3567 (a single cross) were sown in each of 400 Mitscherlich pots containing some gravel and 7 kg sandy soil, each dressed with 2.5 g N, 3.5 g K, 2.6 g P and 1.1 g Mg. Another 0.8 g N, 1.2 g K, 0.3 g P and 0.5 g Mg was applied to the pots 32 and 60 days after sowing in the 2 highest and 2 lowest temperatures respectively.

The pots were divided over four growth cabinets with day/night temperatures of 17/12, 20/15, 25/20 and 30/25 °C (T_1, T_2, T_3 and T_4). Day length was 15 h and irradiance from a mixture of fluorescent tubes (Philips TL 33, 140 W) and incandescent lamps was 120 W/m² (400–700 nm) (= 650 J/cm²/day (400–700 nm)) and 930 J/cm²/day (400–10000 nm). All pots were watered once or twice daily. Plants were sampled successively at the following stages of development.

1. 4th leaf present (pots thinned to 5 plants)
2. 8th leaf present (2 plants per pot)
3. 12th leaf present (1 plant per pot)
4. 16th leaf present (1 plant per pot)
5. ♂ flower present (12 pots per treatment)
6. ♀ flower present (12 pots per treatment)

7–10. every 2 weeks (12 pots per treatment)

Crop density was about 12 plants per m² after the fourth sampling. At sampling, all plants were separated into numbered individual leaves, stem + leaf sheath, and ♂ and ♀ flowers, if present. Leaf blades were analysed for length, dry mass, and sometimes leaf area, whereas from the other organs only fresh and dry mass was measured. Sometimes the bigger leaves were separated into midrib and 'leaf remainder'.

Samples were dried in a forced ventilated oven at 70 °C and were analysed for true digestibility in vitro of organic matter (D_{om}), estimated by the method of VAN SOEST *et al.* (1966) and for cell-wall constituents (cwc) by the method of VAN SOEST and WINE (1967). Digestibility was chosen as a criterion since it is often the limiting factor in animal nutrition and % cwc since it allows closer investigation into the causes of the different digestibilities (D_{om} is generally about 13 percentage units higher than apparent digestibility in vivo of organic matter). From these two criteria digestibility of cell-wall constituents (D_{cwc}) was calculated, as another factor in the D_{om} obtained.

RESULTS

Course of growth

Plant development increased with temperature as expected. However, with 17/12°C, it was not slower than with 20/15°C, presumably because a temperature of 25/20°C was used during the few days before emergence. Plants with 25/20°C grew so tall after the fifth sampling that they had to be moved to a higher growth chamber in which the same photosynthetically active radiation (400–700 nm) was achieved by mercury lamps, which produced much more infrared radiation. This slowed down production and the plants died faster.

Flowering of the male and female inflorescences was poorly synchronized in this single cross, so seed set was poor at all temperatures, much soluble carbohydrate accumulated in the vegetative parts and the percentage of cell-wall constituents declined during later growth.

Figure 1 presents the curves of growth of the plants and leaves against time. This figure shows that the plants grew rapidly at 30/25°C, but their ultimate yield was lowest, whereas at the lowest temperatures growth was rather slow, but ultimate yield high. These results agree with those of HOOPLOT (1972) in maize and of SPIERTZ (1974) in wheat.

Leaf production increased with rising temperature up to 25/20°C, but dropped so rapidly above that optimum that leaf production was least at 30/25°C. Digestibility of organic matter of leaf blades was very high at the beginning, but dropped rapidly during the first 50 days. Later this decrease proceeded more slowly. The percentage of cell-wall constituents was very low initially but increased to a maximum when the last leaf was formed. Thereafter the percentage declined somewhat, presumably because of the accumulation of soluble carbohydrate.

Chemical composition of the final harvest

Table 1 shows the average composition of leaf blade, leaf sheath and stem with the four temperature treatments. Leaf blade had the highest digestibility of organic matter and of cell-wall constituents, and a rather high % cwc. Leaf sheaths had a higher % cwc than leaf blades, but against expectation the stems showed a substantially lower content, attributable to the accumulation of soluble carbohydrate. Digestibility of cell wall and organic matter in leaf sheath and stem were lower than in leaves. But because of the low cell-wall content stem was even somewhat more digestible than leaf sheath.

The effect of temperature on % cwc was smaller in all fractions than in many other trials with temperate and tropical grasses (DEINUM and DIRVEN, 1972, 1975). The effect seemed curvilinear with a maximum at about 25/20°C. Whether this was a pure effect is not yet certain, but of course variation in accumulation of soluble carbohydrate could have masked the real temperature effect. However % cwc of the leaf blades was the same at the fifth sampling with 17/12 and 25/20°C, indicating the absence of a temperature effect on % cwc. This lack of effect may be a characteristic of

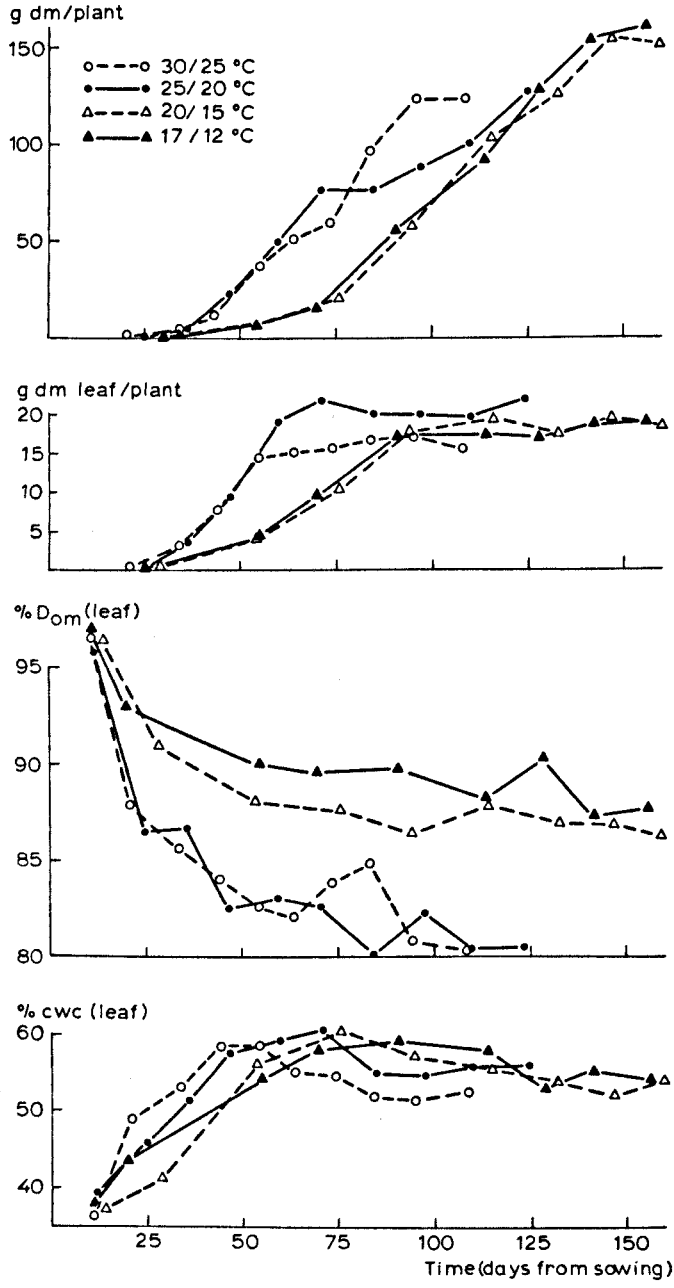

Figure 1. Mass of the whole plant and of the leaf-blade fraction and digestibility and cell-wall content of the latter during the growth of maize at four temperatures.

Table 1. Effect of temperature (T_1–T_4) on percentage cell-wall constituents in organic matter and digestibility in vitro of organic matter (% D_{om}) and of cell-wall constituents (%D_{cwc}) of different parts of maize at the final sampling.

	% cwc				% D_{om}				% D_{cwc}			
	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
Time of final sampling (days)	156	160	124	108	156	160	124	108	156	160	124	108
Leaf	58.7	59.2	62.7	60.8	87.7	86.2	80.4	80.3	79.0	76.7	68.7	67.6
Leaf sheath	64.9	68.0	72.0	68.0	80.4	75.1	67.0	67.8	69.8	63.4	54.2	52.6
Stem	36.3	38.6	44.4	43.1	81.8	78.5	71.7	74.3	49.9	44.3	36.3	40.4

maize as a C-4 plant, in which the low photosynthesis at low temperature does not cause accumulation of soluble carbohydrates and dilution of cell walls, as in temperate grasses at low temperature. Digestibility of cell walls dropped appreciably in all fractions up to 25/20 °C, after which there was not much change to 30/25 °C. Perhaps below optimum temperature for growth, digestibility of cell walls decreases rapidly with rising temperature and above the optimum this decrease proceeds more slowly or vanishes, a hypothesis that is supported by data on *Setaria sphacelata* (DEINUM and DIRVEN, 1976). The effect of temperature on digestibility of organic matter was somewhat greater in this experiment after a constant age of, for instance, 100 days as may be inferred from Figure 1.

Table 1 further suggests that the different tissues of maize give different regression between % cwc and % D_{om}. This may be in line with the poor relationship between net energy and percentage crude fibre in samples from normal fields (STEG and RIEPKEMA, 1975, personal communication).

These results have consequences for practical farming: since soluble carbohydrate may accumulate in the stem, seed formation may not be an essential criterion of productivity and nutritive value of maize grown for silage. This suggestion is supported by later findings (BUNTING, 1975; DEINUM, 1975, unpublished data).

A second aspect is that because of the negative effect of temperature on cell-wall digestibility, the quality of maize silage depends in hot summers as in the United States much more on the accumulation of soluble carbohydrate than in the cool climate of North West Europe.

The effect of leaf number

Figure 2 demonstrates some characteristics of individual leaves with temperature treatments 17/12 and 25/20 °C when reaching mature weight.

Leaf 1 had a very low mass, but later developing leaves showed progressively higher dry mass, so leaf 12 was the heaviest. Thereafter leaf mass dropped till the last leaf developing before the anther (leaf 18).

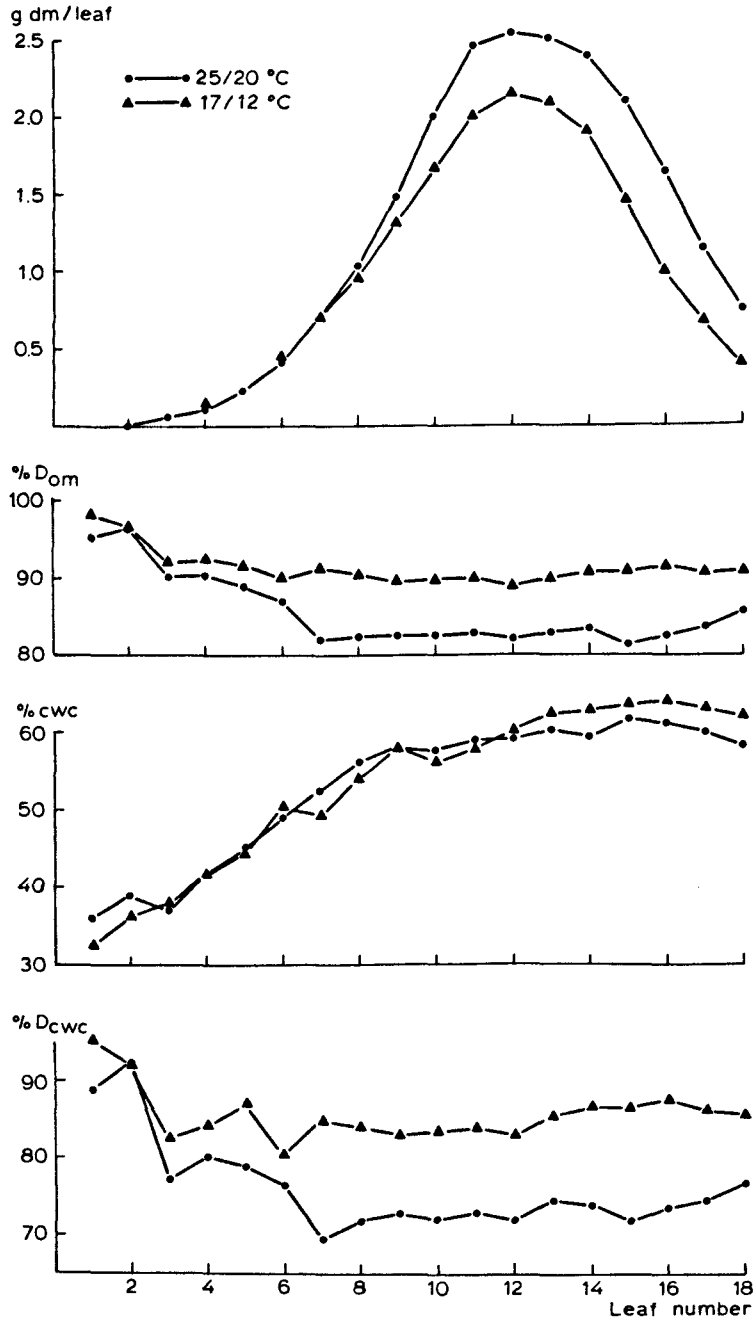

Figure 2. Dry mass, content of cell-wall constituents and digestibility of organic matter and of cell-wall constituents of the successive leaves along the maize stalk at a young mature stage, grown at two temperatures.

Table 2. Effect of temperature (T_1-T_4) on some data of leaf 11-14 of maize in a young mature stage and at final sampling (average of the four leaves).

	Young mature stage				Final harvest			
	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
g dm/leaf	205	183	233	159	223	181	245	169
% D_{om}	89.9	87.5	82.8	81.9	88.6	87.2	80.8	79.5
% cwc	60.7	56.4	59.4	55.7	53.3	53.0	56.8	53.4
% D_{cwc}	84.6	79.4	73.3	70.2	80.1	77.8	69.4	66.4

Temperature had little effect on leaf mass up to leaf 7, but the effect was somewhat peculiar in subsequent leaves (Table 2). Dry mass of the largest leaves was somewhat smaller with 20/15 than with 17/12 °C, but greatest with 25/20 °C, whereas dry mass was least at 30/25 °C. Maybe there has been an unknown stress at 20/15 °C during formation of these leaves, for earlier and later leaves had dry masses between those of 17/12 and 25/20 °C.

Digestibility of organic matter was high in the first leaf and lower in the subsequent ones, reaching a minimum at about leaf 7. Thereafter leaves had about the same low digestibility. With 25/20 °C, digestibility of the largest leaves was about 7 % units less than with 17/12 °C (Figure 2, Table 2). These effects of leaf insertion and temperature can be attributed to effects on percentage and digestibility of cell-wall constituents.

The content of cell-wall constituents of the first leaf was low, about 35 %, but increased with leaf number to a maximum of about 60 % in leaf 15 or 16. With all temperatures there was a slight tendency for specific leaf weight to increase with leaf number from leaf 7. It is not certain whether it is an intrinsic factor to which the cell-wall content is related, since specific leaf weight can change rapidly by accumulation or translocation of assimilates (DEINUM 1975, unpublished data). The higher specific leaf weight of the last leaves may be caused by a greater accumulation of assimilates because they were closer to the light source than the lower leaves.

Temperature had hardly any effect on % cwc as demonstrated in Table 2. Digestibility of cell-wall constituents reacted like digestibility of organic matter, but the differences were greater, showing a decrease up to leaf 7 and a negative effect of higher temperature.

The effect of age on leaf characteristics

As in the former experiment (DEINUM and DIRVEN 1971), leaves reached their final amount of cell walls at the moment of full expansion. Again there was a time lag between leaf expansion and increase in leaf mass. This was more apparent in leaves 9-18 than in earlier leaves, possibly because the later ones remained longer in a cylinder where irradiance was low. Digestibility of organic matter from leaves declined with

Table 3. Decline in digestibility of organic matter in vitro of maize leaves grown at different temperatures, in percentage per day.

	Temperature (°C)			
	17/12	20/15	25/20	30/25
Leaf number				
1- 4	.55	.24	.26	.31
5- 8	.07	.05	.15	.10
9-12	.02	.03	.11	.09
13-16	.03	.03	.04	.14
17-20	.03	.06	.09	.14
5-20	.034	.042	.096	.131

age as expected. However this drop was rather small during the mature life span of the leaf (Table 2 and 3), but greater during leaf expansion, especially at high temperature, perhaps because of progressive lignification of cell walls. Furthermore leaves 1–4 showed a great decrease in digestibility which may be due to abnormal senescence during their very short life span which was much shorter than in the field. (According to GAILLARD (personal communication) the cell walls in such dead brown leaves may become 'horny' and very poorly digestible.) All the other leaves remained alive during the trial, and consequently did not display dying off.

The average effect of ageing (changes during the life span of the leaves) on digestibility was small at low temperature, and much greater at high temperature (Table 3). However, even at high temperature the decline in digestibility was much smaller than in a whole grass crop in which stems were included (DEINUM and DIRVEN, 1975), possibly because of the accumulation of soluble carbohydrates in this trial with maize.

Percentage of cell-wall constituents changed variably with age. In the early leaves (up to 4) there was an increase during their short life span. In later leaves the content increased considerably during leaf expansion, and dropped again until adult leaf mass was attained as in the former trial (DEINUM and DIRVEN, 1971). Thereafter the yield of cell wall in the leaf did not alter any more, but possibly because of the accumulation of soluble carbohydrates the content decreased gradually in the top leaves. However in leaves 6–10 an increase in % cwc was found that was certainly caused by loss of soluble carbohydrate in the low-light environment they were exposed to.

Digestibility of cell walls reacted like digestibility of organic matter, showing a greater decrease at high than at low temperature. However because of the longer life span of the leaves at low temperature, the total decline during life was not much affected by temperature.

Origin of differences between individual leaves

As in the former trial the first leaves were highly digestible and low in cell walls,

whereas later ones were less digestible and richer in cell walls, even when grown at constant temperature. Therefore it is not true that the first leaves were so digestible because they were formed at low temperatures, as supposed before (DEINUM and DIRVEN, 1971).

Consequently the differences between the individual leaves along the stalk of the maize plant must be based on anatomical factors e.g. the larger leaves may need a stronger midrib and stronger sclerenchyma and vascular bundles for support whereas the small leaves do not need much supporting tissue. Because the large leaves have a very thick midrib, this tissue was cut out of the leaf blade at the fifth sampling, weighed and analysed for the same chemical constituents and sometimes for lignin. At that sampling the midrib could be separated from leaves 7–17. There were hardly any differences between the individual leaves, so Table 4 mentions the average data for these leaves with the four temperature treatments.

Contribution of midrib to mass of leaf blade differed slightly with temperature, revealing somewhat higher yield with 25/20 °C and a lower one with 30/25 °C. Midribs were much less digestible than 'leaf remainder', both in organic matter and cell-wall constituents, and they were much higher in cell-wall constituents.

Temperature had no effect on % cwc and only a small negative effect on digestibility of organic matter and cell-wall constituents of 'leaf remainder'. However the effect of temperature on midrib was dramatic, revealing increased contents of cell walls at higher temperatures and greatly diminished digestibilities of cell walls and organic matter. These digestibilities were rather closely related to lignin content.

Further chemical analysis revealed that the cell walls of the midrib contained more cellulose than those from the 'leaf remainder', perhaps because of the greater contribution of sclerenchyma to the fibres of the midrib, as the hemicellulose dominates in the vascular bundles. The contribution of hemicellulose seemed to decrease with temperature contrary to remarks of VAN SOEST (personal communication) that lignin is more connected to hemicellulose than to cellulose. However, it is risky to relate the results of histological research to those of chemical analysis.

Table 4. Effect of temperature on yield and composition of 'leaf remainder' (= leaf – midrib) and midrib of maize (average of leaf 7–17).

	'Leaf remainder'				Midrib			
Temperature (°C)	17/12	20/15	25/20	30/25	17/12	20/15	25/20	30/25
g dm/leaf	1.12	1.12	1.23	.97	.33	.34	.41	.27
% of whole leaf	77.4	76.5	75.0	78.4	22.6	23.5	25.0	21.6
$\%D_{om}$	91.4	90.5	90.8	89.0	77.7	72.5	60.0	57.4
% cwc	56.2	54.4	56.2	55.7	65.9	64.5	69.1	70.8
$\%\ D_{cwc}$	85.6	83.5	84.6	82.4	70.0	60.9	48.7	47.0
% lignin/cwc	3.98	5.28	5.39	–	7.20	7.73	10.14	–

These results have some interesting implications.

1. The effect of temperature on leaf digestibility is almost completely associated with its great effect on midrib digestibility.
2. A great deal of the difference between early and later leaves may be attributable to the size of the midrib.

DISCUSSION AND CONCLUSIONS

Ageing

One of the aspects of ageing of leaves is the general decrease in digestibility of organic matter with time. Since the contents of the cells are almost completely digestible, this decrease in digestibility must originate from the percentage and digestibility of cell-wall constituents.

The attainment of the final amount of cell-wall constituents at the moment of full leaf expansion implies that changes in % cwc of organic matter are associated with accumulation and translocation of assimilates and protein to or from the leaf. Consequently, when an ageing grass leaf in a pasture is progressively shaded by later ones, its % cwc may rise because of both a smaller production of assimilates and removal of protein before death.

Similarly when there are no sufficient sinks for the assimilates as in this experiment with maize, production of assimilates may proceed but translocation is limited, causing a drop in % cwc with ageing. In some instances this drop was even more than 10% in leaves and possibly more than 30% in the stems. This implies that environmental conditions influence ageing of leaves as judges by % cwc, e.g. a rising temperature during ageing promotes respiration and therefore consumption of assimilates, causing an increase in % cwc. This happens in temperate regions in spring, whereas the reverse is true in autumn (cf. DEINUM and DIRVEN, 1971, with *Festuca arundinacea*). That this positive effect of temperature is not so clear in this experiment with maize may be associated with the suggestion of WILSON (1973b) that in tropical grasses like maize the temperature response curve for photosynthesis and growth are about the same, so accumulation of assimilates does not occur at low temperatures, unlike in temperate grasses (DEINUM 1973, unpublished data).

Since changes in % cwc during ageing result from some internal and external factors that have no effect on ageing itself, the pure effects of ageing of leaves are present in the change in digestibility of cell-wall constituents.

As far as we know digestibility of cwc has always shown to decrease with age, as in this trial and former ones (DEINUM and DIRVEN, 1971; DEINUM, 1973, unpublished data; DIRVEN, 1974, unpublished data). This decrease was always significant, although it was rather small (about .03 to .2% unit per day). It varied with environmental conditions. At high temperature it was greater than at low temperature (Table 2), possibly because of a greater lignification. The decrease may be expected to be greater in leaves with a larger proportion of vascular bundles and sclerenchyma than

in the leaves with a smaller proportion, because parenchyma is not lignified and the other two tissues can be lignified extensively (KAWAMURA *et al.*, 1974). These ideas are supported by the finding of MOORE and MOTT (1973), who discovered a minute effect of ageing in a genotype of *Hemarthria* that contained a very small amount of vascular bundles compared to genotypes with a much greater amount.

Irradiance or plant density had no effect on this ageing, for no interaction with age was found in an experiment with 3 and 30 plants per m^2, although the leaves of the latter weighed only half as much as those of the former (DEINUM, 1974, unpublished data).

From this information it may be clear that digestibility of organic matter in leaves may decrease with age to various extents because of different reactions of percentage and digestibility of cell-wall constituents. For instance, with a rising temperature as in spring in temperate regions, % cwc will increase and digestibility of cwc will decrease rapidly, whereas with a decreasing temperature as in autumn, ageing as judged by D_{om} may be very small or even positive if the decrease in digestibility of cwc is counterbalanced by a sharp drop in % cwc.

Effect of leaf number

The trial with maize revealed that the first leaves had least % cwc and highest % D_{om}, whereas later leaves were less nutritious, having more cell-wall constituents and lower digestibilities. Similar results were found by DIRVEN (1974, unpublished data) in some tropical grasses, by WILSON (1971) in *Panicum maximum*, and HAGGAR and AHMED (1971) in *Andropogon gayanus*. Since this phenomenon is found also in the temperate species *Avena sativa* and *Lolium multiflorum* for % cwc (DEINUM, 1973, unpublished data) it may possibly be considered a general feature of grasses.

What could cause this phenomenon? Much of it must originate from morphological differences, as indicated by Table 4, which shows that the midrib has much more cwc than does 'leaf remainder'.

Nevertheless, the midrib does not explain all the difference, for % cwc of the 'leaf remainder' of leaves 7–17 was still much higher than % cwc of the whole leaves 1–4, whereas % D_{om} and % D_{cwc} of the leaves 1–4 was still substantially higher than of 'leaf remainder' of the leaves 7–17. Visual examination of the leaves revealed that the vascular bundles and midrib were hardly detectable in leaf 1 and 2, but these tissues were clearly present in the 'leaf remainder' of leaves 7–17. Consequently anatomical analysis of the leaves from a later trial showed that the relative area of xylem and sclerenchyma of the first leaves was very low, but much greater in the later ones. In addition incubation in vitro of intact parts of the leaves showed complete digestion of the parenchyma cell walls of all leaves, but xylem, epidermis and sclerenchyma seemed to remain undigested. These findings accord very well with those of MONSON *et al.* (1972) in *Cynodon dactylon* and *Pennisetum typhoides* and of KAWAMURA *et al.* (1974) in *Oryza sativa*.

Since parenchyma is not lignified, temperature and other external growth factors

like irradiance and nitrogen supply have no detrimental influence on its digestibility, but temperature stimulates lignification in supporting tissues like sclerenchyma and vascular bundles, causing a drop in digestibility of these tissues. Therefore a higher temperature decreases digestibility of leaves and stems to an extent releated to the proportion of cell walls present in non-parenchymatic tissues of grasses, and probably also of legumes.

A factor like plant density or the reverse (irradiance) did not seem important in this, for in the greenhouse experiment with maize at 3 and 30 plants per m^2, the low plant density resulted in only slightly lower % cwc and % D_{cwc}, though the leaves were about twice as heavy as those from the high plant density. Neither was there an effect of plant density on % D_{cwc} and % cwc of particular leaves in the succession. The sole effect of plant density was that % cwc of the 'leaf remainder' and the midrib at low density was about 4% units lower, which was certainly caused by a greater accumulation of soluble carbohydrate.

Consequently, the enormous impact of the lignified tissues on digestibility suggests that is worthwhile to breed for higher digestibility of forages by selecting for a smaller contribution of vascular bundles and sclerenchyma fibres. Some progress has already been made in this. So MULLER *et al.* (1971, 1972) and BARNES *et al.* (1971) report on brown midrib mutants of maize that are more digestible and less lignified than the normal variety, and MOORE and MOTT (1974) describe a *Hemarthria* species with a smaller quantity of vascular bundles that is high in digestibility, low in lignin and hardly deteriorates with ageing.

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HEMICELLULOSE DEGRADATION IN THE RUMINANT

R. F. H. DEKKER

Biologisches Institut II, Lehrstuhl für Biochemie der Pflanzen, der Universität, Freiburg i. Br., 7800. 9–11 Schänzlestrasse, West Germany

SUMMARY

The degradation of the plant cell-wall polysaccharide, hemicellulose, in the ruminant is discussed. These polysaccharide consituents intact in the plant are incompletely digested within the rumen due to their encrustation with lignin which acts as a physical barrier to the rumen bacteria and protozoa, and their enzyme systems. Other factors discussed thought to be involved in the incomplete digestion of these polysaccharide constituents include the chemical heterogeneity of the latter, and the influence of the kind of microbial population dominant within the rumen following preconditioning of the animal. *In-vitro* rumen digestion experiments have indicated that the latter; i.e., the type of 'digesters' e.g., bacteria, or protozoa, having the ability to degrade and/or utilize hemicellulose, are more likely to influence fermentation of hemicellulose, rather, than chemical heterogeneity of the polysaccharide constituent. The enzyme systems in rumen bacteria and protozoa responsible for the hydrolysis of different hemicelluloses include the hemicellulases, e.g., the galactanases, mannanases and xylanases, and the following glycosidases: α- and β-D-galactosidases, β-D-mannosidases, α-L-arabinofuranosidases, and β-D-xylosidases. The synergistic actions of these enzymes in degrading hemicellulose are also discussed.

INTRODUCTION

The enzymes within rumen bacteria and protozoa responsible for the degradation of plant hemicellulose are the hemicellulases. The latter as a group of enzymes have been defined and classified according to their substrates (DEKKER and RICHARDS, 1976). The hemicelluloses are an indefinite group of polysaccharides which have been variously defined, e.g., as soluble in alkali that are associated with cellulose of the plant cell-wall. They are usually classified according to the sugar residues present, e.g. L-arabinan, D-galactan, D-mannan and D-xylan. However, they do not occur as homoglycans, but rather as heteroglycans, containing different types of sugar residues, often as short appendages linked to the main backbone chain (ASPINALL 1959, 1970; and TIMELL, 1964 and 1965); their general structures are summarized in figure 1.

Hemicellulases or hemicellulose-degrading enzymes, are glycan hydrolases (EC 3.2.1) which specifically degrade the hemicelluloses as defined above; they include the L-arabinanases, D-galactanases, D-mannanases and the D-xylanases (DEKKER and RICHARDS, 1976). The pathways and enzyme systems responsible for the degradation,

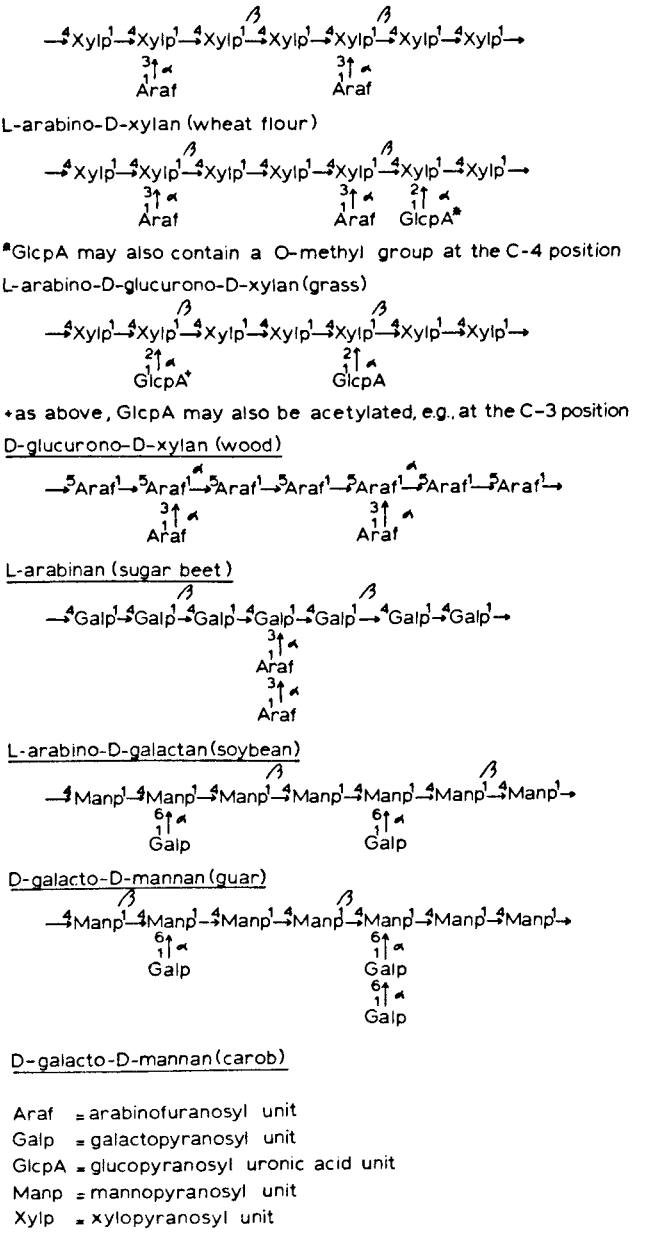

Fig. 1. The structures of some common plant hemicelluloses.

or digestion, of the hemicellulose components of the plant cell-wall by hemicellulases in rumen bacteria and protozoa, and the fermentation of the monosaccharides arising from their hydrolysis to volatile fatty acids are shown in figure 2. The presence of hemicellulolytic enzymes in the mucosal lining of the gastro-intestinal tract have not

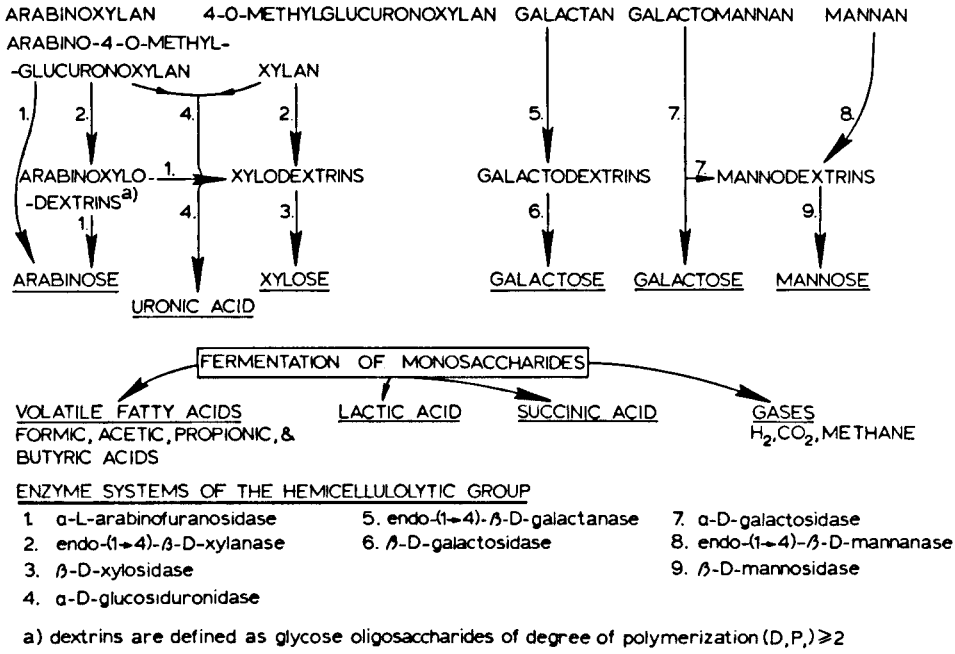

Figure 2. Enzyme systems in rumen bacteria and protozoa responsible for the digestion of hemicelluloses in the ruminant.

been detected, and the ruminant therefore relies solely upon the microbial flora and fauna within the rumen for the digestion of these polysaccharides.

In vitro experiments utilizing crude cell-free rumen bacterial and protozoal enzyme preparations have shown (BAILEY and GAILLARD, 1965; BAILEY and GAILLARD, 1969; HOWARD *et al.*, 1960) that hemicellulases rely upon the synergistic action of glycosidases, e.g., α-L-arabinofuranosidases (EC 3.2.1.55), α- and β-D-galactosidases (EC 3.2.1.22 and 23, respectively), β-D-mannosidases (EC 3.2.1.25) and β-D-xylosidases (EC 3.2.1.37). These enzymes are also capable of hydrolysing the short chain of monosaccharide appendages from the main backbone chain of hemicelluloses. In the rumen ecosystem, it is therefore necessary to include the aforementioned glycosidases in the group of hemicellulose-degrading enzymes, as they are responsible for

1. 'Opening up' the glycan chain prior to the attack by the hemicellulases, and,
2. Hydrolysing the oligosaccharides resulting from attack by endohemicellulases, to monosaccharides which, in turn, are utilized by the 'microbial fermenters' in the rumen (see figure 2).

In discussing the degradation of plant hemicelluloses by the ruminant it is necessary to discuss two aspects which are involved in their breakdown, viz.,

1. The digestion of hemicelluloses *in-situ* in the plant within the rumen, and,
2. The enzyme systems responsible for their degradation.

DIGESTION OF HEMICELLULOSES IN THE RUMEN

Hemicelluloses constitute 10–30% of the roughages commonly used in ruminant rations (GAILLARD, 1966), and as such form a major proportion of the total feed carbohydrate. Their content has been reported (GAILLARD, 1966; and SULLIVAN, 1966) to be greater in grasses than in legumes, and has been shown (GAILLARD, 1962, and WAITE *et al.*, 1964) to progressively increase as the plant matures. *In-situ* hemicelluloses in the plant, are only partially digested by the ruminant, and their *in-vivo* digestibilities in pasture grasses and legumes have been reported to range from 30–80% (DEKKER, 1972).

GAILLARD (1966) and WAITE *et al.*, (1964) have shown that the apparent digestibility of hemicellulose decreases as the plant matures and this phenomenon has been shown to correlate with the increased degree of lignification of the plant which physically protects the digestion of the enclosed cell nutrients. GAILLARD (1966), SULLIVAN (1966) and BALWANII *et al.*, (1969) have also reported that grass and legume hemicelluloses differed in their digestibilities, and they suggested that this may be due to a difference in the chemical heterogeneity of the xylans, which are the principal hemicellulosic components in these pasture forages. WAITE *et al.*, (1964) found that during growth the xylan fraction which contained uronic acid constituents became progressively less digestible. Furthermore, they and DEKKER *et al.*, (1972, 1973a) found that the neutral glycan constituents of the hemicelluloses were more digestible than xylan or the uronic acid. This has also been demonstrated by BAILEY (1967), who showed that arabinose and galactose constituents were removed from the hemicellulosic molecule at a faster rate than xylose during the course of digestion in the rumen.

MACRAE and ARMSTRONG (1969) found that when all or a major part of the ration fed to sheep was hay, 93–97% of the digestible 'hemicellulose' (measured as non-glucose reducing polymer) was digested in the reticulo-rumen, and when the rations contained low roughagehigh cereal diets, 71–85% of the digestible 'hemicellulose' disappeared before the small intestine, and appreciable amounts were digested in the caecum and colon. The high starch diet may in some way have suppressed the secretion of hemicellulases in favour of amylolysis by the microbial population in the rumen and thereby resulted in lower digestibilities of this fraction. BAILEY and MACRAE (1970), however, found that little hemicellulose escaping ruminal digestion disappeared in the caecum of sheep fed red clover (*Trifolium pratense*) hay roughages, and they showed that the resistance to further digestion was due to lignin forming a physical barrier to the microorganisms from both the rumen and caecum, and the intact hemicellulose. Furthermore, in *in-vitro* experiments utilizing both rumen and caecal microbial extracts, they were able to demonstrate that both extracts were capable of hydrolysing isolated hemicellulose, and delignified digesta particulate hemicellulose, but were not, to any extent, capable of further hydrolyzing the hemicellulose in undelignified digesta particles from various parts of the post-ruminal digestive tract.

In studies on ruminant digestion of polysaccharide constituents of tropical pasture

plants, viz. the legume, *Stylosanthes humilis* and the grass, *Heteropogon contortus*, it was shown by DEKKER *et al.*, (1972, and 1973a, respectively) that the hemicellulose constituents were incompletely digested *in vivo* (30–40%). Furthermore, they showed (DEKKER and RICHARDS, 1973b) that when sugar cane bagasse (the fibrous material resulting after crushing of sugar cane) was delignified by several different delignifying processes, the *in-vitro* dry matter digestibility and the *in-vitro* digestibility of the monosaccharide constituent of hemicellulose increased several fold. Their results support the work of BAILEY and MACRAE (1970) and conclude that incomplete digestibility of plant *in-situ* hemicelluloses is due to inaccessibility of the rumen flora and fauna to these constituents, because of their encrustation with lignin which acts as a physical barrier to these microorganisms.

With respect to chemical heterogeneity of the hemicelluloses it has been shown (GAILLARD, 1965) that these polysaccharide constituents in grasses and legumes are composed of a mixture of different polymers, viz., linear and branched, which are separated by complexing with iodine. Distinct structural differences have been found (BAILEY and GAILLARD, 1965) between corresponding polymer fractions from each group, esp., in the linear xylan from the hemicellulose A fraction, and in the branched polymer from hemicellulose B. The action of rumen microbial enzymes on these isolated polymer fractions from grass (*Lolium perenne*) and red clover (*T. pratense*) showed that the polymers from grass were hydrolysed at a higher rate than the corresponding polymers from clover, and that the branched B polymers from both species were more resistant to hydrolysis than the linear polymers, BAILEY and GAILLARD, (1965) and GAILLARD *et al.*, (1965). Of the linear polymers, the B fraction was hydrolysed most rapidly and explains why hemicellulose B is more digestible than hemicellulose A in red clover (see BAILEY and MACRAE, 1970). The resistance to hydrolysis of the branched B polymers by the rumen microorganisms was suggested to be due, most probably, to the high content of uronic acid in this polymer.

Contrary to the observation of BAILEY and coworkers (1965, 1970), BEVERIDGE and RICHARDS (1973a, b) in using the tropical grass, *Heteropogon contortus*, have shown, that incomplete digestion of hemicellulose was due to lignin rather than to a structural difference between hemicellulose components. Samples of this grass, after 72h digestion in the bovine rumen, showed no significant difference in composition of hemicellulose B from that of the fresh sample. Furthermore, this trend was also observable in the linear and branched fractions of hemicellulose B. In all cases the branched hemicellulose fraction was shown to contain more arabinose and uronic acid than the corresponding linear fractions, and in this respect its composition is very similar to that reported for the temperate grass, *L. perenne* (BAILEY and GAILLARD, 1965).

The similarity in composition of the hemicelluloses arising from the tropical grass, *Setaria splendida*, and *L. perenne* before and after digestion (faeces fraction) has also been demonstrated by FORD (1973). However, the lower digestibility of *S. splendida* (DMD of 61.4%, vs 76.1% for *L. perenne*) could not be accounted for in terms of their cell-wall polysaccharides and is thought to be due to some gross structural difference

between the two grasses. BEVERIDGE and RICHARDS (1973b) further showed that there was no significant difference in the rates of hydroysis of the hemicellulose fractions examined. Previous results (e.g., BAILEY and coworkers, 1965, 1970) suggesting such differences, can be attributed to the presence of a readily digested glucan in the linear hemicellulose fraction. DEKKER and RICHARDS (1973b) have also demonstrated that the hemicelluloses surviving digestion in the rumen, when isolated, were further capable of being attacked by two highly purified xylanases of the fungal phytopathogen, *Ceratocystis paradoxa*. The faeces hemicellulose was hydrolised to the same extent, liberating similar degradation products, as the hemicellulose of the original fodder, and confirms the conclusion that resistance to digestion is due to physical protection by lignin rather than a structural difference.

The different observations of the aforementioned groups of workers may be interpreted in terms of the type of microbial species inhabiting the rumen acquired from preconditioning of the animals on different rations, e.g. the preference of cellulose-digesters to that of hemicellulose-digesters, or vice versa; or combinations of the two which show activation of inhibition of digestion. The complexity of overall rumen microbial degradation and utilization of hemicellulose has been demonstrated by DEHORITY (1973) who observed (in *in-vitro* rumen experiments) a synergistic effect when pure cultures of non-cellulose and cellulose digesting rumen bacterial strains were combined, (table 1). This effect was also observable in the fermentation of pectin

Table 1. The effect of pure cultures of rumen bacterial cellulose and hemicellulose digesting strains and their combination, on the degradation and utilization of intact forage and isolated hemicellulose[a].

Bacterial organism	Intact bromegrass substrate Stage of growth (boot)		(bloom)		Intact fescue grass substrate		Isolated fescue grass hemicellulose substrate	
	De-graded (%)[b]	Utilized (%)[c]	De-graded (%)	Utilized (%)	De-graded (%)	Utilized (%)	De-graded (%)	Utilized (%)
Bacteroides ruminicola H8a (a hemicellulotytic strain)	5	6	5	6	3	2	82	80
Buturivibrio fibrisolvens H10b	52	41	33	27	45	38	88	84
Ruminococcus flavefaciens B34b (cellulolytic strains)	78	0	61	0	67	3	89	0
Combination of strains								
H8a B34b	84	80	70	67	69	68	94	87
H10b B34b	81	70	66	59	67	65	91	88

[a] from Dehority (1973).

[b] Degradation is defines as the solubilization, or depolymerization of the hemicellulose into 80% ethanol-soluble oligosaccharides.

[c] Utilization (or fermentation) of these intermediates[b].

and cellulose by rumen bacteria. In the case of isolated hemicellulose (table 1) however, the synergistic effects were minimal indicating that either chemical isolation of the hemicellulose or solubilization of the hemicellulose in the intact forage by a non-utilizing strain will allow extensive utilization of hemicellulose by those xylan-digesting strains which have limited ability or are incapable of attacking the hemicellulose in its native state. Furthermore, DEHORITY (1973) showed that the differences in the ability of a particular organism to degrade and utilize the isolated hemicelluloses does not appear to be directly related to hemicellulose composition. He observed no marked differences in the initial rate of isolated hemicellulose degradation between the hemicellulose utilizing and non-utilizing cellulolytic strains; the rates appeared more closely related to the amount of hemicellulose that each particular cellulolytic strain could degrade rather than the ability of the organism to utilize the substrate. DEHORITY (1967) also showed that there was no build up of partially-hydrolysed 'hemicellulose' residues, (i.e., indigestible) varying in gross composition from the original hemicellulose, which indicates that the synergism of the different strains and species of rumen bacteria in the *in-vitro* system are capable of almost complete fermentation of hemicellulose.

HEMICELLULOSE-DEGRADING ENZYMES (HEMICELLULASES)

Occurrence

Rumen bacteria (BAILEY and MACRAE, 1970; CLARKE *et al.*, 1969; DEHORITY, 1967; 1973; GAILLARD *et al.*, 1965; and HOWARD *et al.*, 1960) and protozoa (ABOU-AKKADA *et al.*, 1963; BAILEY and CLATKE, 1963; BAILEY *et al.*, 1962; BAILEY and GAILLARD, 1965; and NAGA and EL-SHAZLY, 1968), and caecal bacteria from sheep (BAILEY and MACRAE, 1970) produce enzymes capable of liberating arabinose from arabinoxylans and are most probably α-L-arabinofuranosidases. CLARKE *et al.*, (1969) showed that the cell-free enzyme preparation from the rumen bacterium *Butyrivibrio fibrisolvens* was capable of releasing arabinose from wheat flour arabinoxylan and sugar beet L-arabinan, but the appearance of only small amounts of arabinose in the enzymic hydrolysate of the latter suggests the action of an arabinosidase. Rumen enzymes capable of hydrolysing the backbone chain of L-arabinan have not yet been detected. The same bacterium was also found to be capable of hydrolysing larch and lupin (1→4)-β-D-galactan.

Mannanases have been reported (WILLIAMS and DOETSCH, 1960) to be produced by rumen bacterial species belonging to the genera: *Streptococcus*, and by rumen protozoa (BAILEY and GAILLARD, 1969). The former were shown to be extracellularly produced, whilst the latter were of intracellular origin.

Most of the work on rumen hemicellulases has been concerned with the xylanases, since their substrate, the xylans, constitute the largest proportion of the hemicelluloses in pasture plants. Xylanases have been detected in several rumen bacterial and protozoal species, and in ruminant caecal bacteria and their sources are shown in table 2.

Table 2. Sources of rumen microbial xylanases.

Organism	Reference
Bacteria	
Bacillus firmus	Inaoka (1961)
Bacteroides sp.	Hungate (1966); Walker (1961)
Bacteroides amylogenes	Howard et. al., (1960); Hungate (1966)
Bacteroides ruminicola	Dehority (1967)
Bacteroides succinigenes	Dehority (1967)
Butyrivibrio Sp.	Howard et al., (1960); Kock and Kistner (1969)
Butyrivibrio fibrisolvens	Clarke et al., (1969); Gaillard et al., (1965); Howard et al., (1960)
Clostridium sp.	Kock and Kistner (1969)
Ruminococcus albus	Dehority (1967); Kistner and Gouws (1964); Kock and Kistner (1969)
Ruminococcus flavefaciens	Dehority (1967); Kock and Kistner (1969)
Mixed rumen bacteria	Bailey and MacRae (1970); Gaillard et al., (1965); Howard (1951)
Mixed caecal bacteria (sheep)	Bailey and MacRae (1970)
Protozoa	
Epidinium ecaudatum (Crawley)	Bailey et al., (1962, 1965)
Entodinium sp.	Bailey and Clarke (1963); Bailey and Gaillard (1965)
Eremoplastron bovis	Bailey and Clark (1963), Bailey and Gaillard (1965)
Eudiplodinium medium	Naga and El-Shazly (1968)
Polyplastron multivesiculatum	Abou-Akkada et al., (1963)
Mixed rumen micro-organisms	Bailey and MacRae (1970); Beveridge and Richards (1973b); Walker (1967)

Extraction of intra-cellular xylanases

Xylanases of rumen microbial origin are mostly of intracellular origin, but lately there have been reports of xylanases produced extracellularly (BEVERIDGE and RICHARDS, 1973b; DEHORITY, 1973; and MORRISON, 1975). Intracellular xylanases have been isolated from rumen bacteria and protozoa by rupturing the cell wall of these organisms, e.g., by grinding with Ballotini glass beads (BAILEY and GAILLARD, 1969; CLARKE *et al.*, 1969; GAILLARD *et al.*, 1965); a French press (BAILEY and MACRAE, 1970); or sonication (BEVERIDGE and RICHARDS, 1973b).

Purification of hemicellulases

Hemicellulases of rumen microbial origin have not yet been purified to homogeneity, and most of the work in this field has utilized crude or partially purified enzyme preparations. Such studies suffer from the disadvantage that the observations may results from the action of more than one enzyme component.

Mode of Action

As mentioned previously, in a discussion of the attack of hemicellulases on hemicellulose in the rumen microbial system, it is necessary to include the hydrolytic action of the glycosidases, (see figure 2). In this way, the polysaccharide constituents are more completely degraded to monosaccharides, which are subsequently fermented.

Two types of polysaccharide-degrading enzymes are known to exist, viz., exo and endo. An exo-enzyme is defined as one that degrades the polysaccharide by successive removal of terminal glycose or oligo-saccharide units, and proceeds in a stepwise manner usually removing the glycose unit from the non-reducing end of the polymeric chain.

Exo-glycanases are difficult to distinguish from glucosidases since both are capable of degrading oligosaccharides of D.P. >2, but by definition, only the former should attack the glucan. The two types of enzymes can be readily distinguished by NMR spectral analysis, e.g., see GORIN *et al.*, (1969), which determines the configuration of the glycose residue released. Retention of configuration indicates a glycosidase enzyme, while inversion denotes an exo-enzyme.

Hemicellulases of an exo-enzyme nature have not yet been identified in rumen microbial species.

Endo-hemicellulases are the most common source of hemicellulases produced by rumen microorganisms. An endo-enzyme attacks the polymer substrate at random. During the initial stages of degradation more than one molecule of enzyme may bind to one molecule of substrate causing multiple scission of the polymer. During this and subsequent stages of hydrolysis the polymer is progressively degraded into shorter fragments, each of which serve as the substrate for the next stage of hydrolysis, and so on, until non-degradable products are formed.

Degradation of xylans

The group of enzymes produced by rumen bacteria and protozoa capable of degrading arabinoxylans, arabino-4-O-methylglucuronoxylans, 4-O-methylglucuronoxylans, glucuronoxylans and xylans from terrestrial plant and marine algal species include the α-L-arabinofuranosidase, β-D-xylosidase or xylobiase, 'xylodextrinase' and (1→4)-β-D-xylanase (EC 3.2.1.8). Also to be included in this group is α-D-glucosiduronidase which is capable of hydrolysing the α-D-(1→2)-linked D-glucopyranosyluronic groups of the glucuronoxylans. However, enzymes with this type of specificity have not yet been detected in rumen microorganisms.

The aforementioned enzymes have been isolated from pure culture isolates of the rumen bacteria: *Bacillus firmus*, *Bacteroides amylogenes*, *Butyrivibrio fibrosolvens* and *Ruminococcus flavefaciens*, and the rumen ciliates: *Epidinium ecaudatum*, an *Entodinium* species, *Eudiplodinium medium* and *Polyplastron multivesiculatum* (see table 2).

β-D-Xylosidase hydrolyses xylobiose (X_2) and xylose oligosaccharides of D.P. $\geqslant 3$ resulting from endo-xylanase action on xylan, to D-xylose. They do not attack xylan. BAILEY and GAILLARD (1965) isolated and partially purified a 'xylodextrinase' from

cell-free extracts of *E. ecaudatum* which was found to degrade xylose oligosaccharides of D.P. 3–5 to xylose and X_2. This enzyme was separated from other hemicellulases by fractionation on Sephadex G-100 and DEAE-cellulose; it did not degrade X_2 or xylan. The α-L-arabinofuranosidases have been shown to hydrolyse the α-L-(1→3)-linked arabinose branch-points of arabinoxylan, (BAILEY and MACRAE, 1970; HOWARD *et al.*, 1960).

The endo-xylanases of rumen microbial origin degrade xylan, arabinoxylan, hemicellulose B, and xylose oligosaccharides of D.P. ≥3 mainly to X_2 and xylose; and X_2 is not attacked. Arabino-xylose oligosaccharides of D.P. >2, in addition to the xylose oligosaccharides, have also been found in enzymic hydrolysates of arabinoxylan employing rumen bacterial and mixed microbial cell-free extracts, (HOWARD, 1957; and BEVERIDGE and RICHARDS, 1973b).

The xylanases have been shown (BAILEY and GAILLARD, 1965; BAILEY and MACRAE, 1970; and HOWARD *et al.*, 1960) to have a higher affinity for linear xylans than for the branched hemicelluloses, viz., wheat flour arabinoxylan and a branched B fraction from red clover. The presence of arabinose, galactose and uronic acid substituents on the xylan backbone prevented the complete degradation of these polymers by xylanase. Furthermore, they demonstrated that the xylan was not effectively hydrolysed until the side-group substituents had been mostly removed, e.g., by the action of an arabinosidase which was found to precede the action of the xylosidase (s) and xylanase(s) (BAILEY and GAILLARD, 1965). This then demonstrates the synergistic manner in which rumen microbial hemicellulases degrade the hemicellulosic substrates.

This type of hemicellulase system is therefore very different from that described for the phytopathogens *Ceratocystis paradoxa* and *Cephalosporium sacchari* in which the xylanase preferentially attacked the branched hemicellulose, DEKKER and RICHARDS, 1975a, b; 1976, and DEKKER *et al.*, 1975d.

Rhodymenan, an algal xylan containing both β-D-(1→3)-and β-D-(1→4)-linked xylose residues isolated from *Rhodymenia palmata*, has also been found to be hydrolysed by xylanases of rumen bacteria yielding xylose, a series of β-D-(1→4)-linked xylose oligosaccharides of D.P. >2, and a series of xylose oligosaccharides containing both (1→3)- and (1→4)-linkages (HOWARD, 1957).

The arabino-xylose oligosaccharides isolated by BEVERIDGE and RICHARDS (1973b) were characterized by DEKKER and RICHARDS (1975a, b, c), using two purified endo-xylanase isolated from *C. paradoxa*, to constitute a series of oligosaccharides of D.P. 4–6 in which the arabinose substituent was found to be on the non-reducing xylose unit. One of these oligosaccharides, viz., AX_3, is now thought to be: O-α-L-Araf-(1→3)-O-β-D-xylp-(1→4)-D-xylp-(1→4)-D-xylp (see DEKKER and RICHARDS, 1975c).

Degradation of mannans

Various terrestrial plant and seed mannans, e.g., guaran (a galactomannan from *Cyamopsis tetragonolobus*), ivory nut (a β-D-(1→4) mannan from *Phytelephas macro-*

carpa) and a galactoglucomannan from *T. pratense*, have been shown by BAILEY and GAILLARD (1969) to be degraded by cell-free extracts of mixed rumen protozoa. Ivory nut mannan yielded a series of mannose oligosaccharides of D.P. $\geqslant 2$ and mannose indicating the presence of an endo-mannanase, EC 3.2.1.78. Guaran, upon attack, liberated initially galactose, followed later by a series of mannose oligosaccharides and mannose, indicating that the main backbone chain of this branched hemicellulose was not attacked until most of the α-D-galactopyranosyl residues were removed by the action of an α-galactosidase, c.f., with the attack on arabinoxylans. Enzymic hydrolysates of clover mannan yielded only monosaccharides and the lack of oligosaccharides suggests the complexity of this polysaccharide which is not attacked because of the side chain substituents. The group of hemicellulases in rumen oligotrich protozoa, (holotrichs do not ingest plant particles), responsible for the complete degradation of the mannans therefore include the α-D-galactosidases, β-D-mannosidases and (1→4)-β-D-mannanases (see figure 2).

Rumen bacteria, obligative non-motile species of *Streptococci*, have also been found to produce mannanases (extra-cellular) when grown on media containing guaran (WILLIAMS and DOETSCH, 1960). Although galactose, mannose of mannose oligosaccharides could not be demonstrated in enzymic hydrolysates of galactomannan, WILLIAMS and DOETSCH (1960) were able to show, from experiments measuring reducing sugars released during hydrolysis, and in the change in viscosity of the polymer substrate, that their mannanase preparation was of the endo-type.

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THE RELATIONSHIP BETWEEN LIGNIN AND CARBOHYDRATE IN THE HEMICELLULOSE A, B AND C FRACTIONS EXTRACTED FROM LUCERNE AND WHEATSTRAW WITH ALKALI

ANTHONY J. GORDON[1, 2, 3] and BLANCHE D. E. GAILLARD

Department of Animal Physiology, Agricultural University, Wageningen, The Netherlands

SUMMARY

Hemicellulose fractions A, B, and C were extracted from wheatstraw and lucerne with KOH without prior delignification. Lignin had very little effect on the carbohydrate composition of the wheatstraw fractions but it prevented clear separation of the lucerne fractions, perhaps linking them together. However, gelfiltration studies and the solubility of the lignins and hemicellulose in the alkali suggested a greater frequency of lignin-carbohydrate links in wheatstraw. With the wheatstraw, 78%, 8% and 14% of the total lignin extracted was revovered in the A, B and C fractions respectively. For the lucerne the values were 46%, 17% and 37%. The A fractions, especially that from wheatstraw, contained a carbohydrate rich in arabinose which was soluble in ethanol and may have been covalently linked to the lignin. More lignin was found in the linear B polymers than in the branched B. The lucerne branched B showed two carbohydrate peaks on Sephadex G-100 chromatography but the linear B gave only one. The lucerne C fraction gave two carbohydrate peaks on Sephadex G-50 but only one on G-100. The wheatstraw C showed two carbohydrate peaks on G-100, the second probably representing carbohydrate linked to lignin.

INTRODUCTION

The extraction and separation of plant hemicelluloses into fractions is usually preceded by a delignification procedure so that clear cut separation of the fractions can be obtained. Lignin probably interferes with the separation through covalent linkages with carbohydrate, perhaps joining one hemicellulose fraction with another or preventing the complete extraction of each fraction. A comparison of the carbohydrate composition and lignin content of each fraction obtained from plants with and without prior delignification might indicate the nature and frequency of such linkages.

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[3] Formerly Research Fellow, Department of Animal Physiology, Agricultural University, Wageningen.

Since the carbohydrate composition of the A and B fractions from delignified plants has been well established for members of the Leguminosae and Gramineae (GAILLARD, 1965) one representative from each family was chosen to determine the composition of these fractions without prior delignification. They were *Triticum vulgare* and *Medicago sativa*.

Although little is known of the C fraction, BLAKE *et al.* (1971) concluded that it consisted mainly of low molecular weight lignin and carbohydrate with sodium silicate derived from silica in the plant. The presence of lignin has not been established by these authors. It was proposed to establish this fact in this work. The proportion of lignin associated with each fraction is important because this compound greatly determines the extent of digestion by ruminants of the hemicellulose. In the absence of lignin each carbohydrate fraction is probably easily digested by rumen hemicellulases (BEVERIDGE and RICHARDS, 1973; BAILEY and MACRAE, 1970) although at different rates (BAILEY and GAILLARD, 1965; GAILLARD *et al.*, 1965). Furthermore, the lignins from Leguminosae seem to have less effect on the digestibility of hemicellulose than do the lignins from Gramineae (GORDON, 1975).

EXPERIMENTAL

Alkali extraction

The air-dry ethanol:benzene (1:2) extracted sample (50 g) was extracted by shaking with KOH (10% (w/v), 500 ml) under N_2 for 16 h in a polythene bottle fitted with a screw cap. The extract was then filtered and washed free of the residue with water and centrifuged to remove any particulate matter. The supernatant was acidified with acetic acid (50%) to precipitate the A fraction. After cooling in a refrigerator for at least 3 h, it was centrifuged and the supernatant decanted into 4 volumes of ethanol to precipitate the B fraction, which was collected by centrifuging. The ethanol in the supernatant from this step was evaporated under reduced pressure and the remaining C fraction subjected to dialysis for 5–7 days under running tap water. It was then freeze-dried. The A and B fractions were purified by redissolving in KOH (10%) and reprecipitating with acetic acid and ethanol as described above.

In an attempt to separate the lignin from hemicellulose in the A fraction, a sample (1 g) was dissolved in KOH (5%, 35 ml) and the solution poured into ethanol (150 ml). After centrifuging, the supernatant (after removal of the ethanol) and precipitate were dialyzed against running tap water for 5–7 days and freeze-dried. They were called soluble A and insoluble A representing mainly lignin and mainly hemicellulose respectively.

The total B fraction was separated into linear B and branched B fractions by iodine precipitation (GAILLARD, 1965).

The residue from the alkali extraction procedure was thoroughly washed with water, freeze-dried and weighed.

Analysis of total hemicellulose and lignin

These cell wall components were determined in the original unextracted material and in the residue from the alkaline extraction. The sample was hydrolyzed for 3 h with boiling N H_2SO_4. After filtration, the filtrate was made to volume, the sugar determined in it by reaction with ferricyanide (HAGEDORN and JENSEN, 1923) and calculated as anhydroxylose. Uronic acids were determined (DISCHE, 1947) and, as anhydro uronic acid, added to the anhydroxylose to give total hemicellulose. The residue was dried with acetone at room temperature, treated with H_2SO_4 72% at 20°C ± 2 for 4 h and after dilution to 2N, further hydrolyzed (6 h) in a boiling waterbath. After filtration the lignin in the residue was determined as the weight loss upon ashing'

Carbohydrate analysis in A, B and C fractions

Total carbohydrate in each fraction was determined by the phenol-sulphuric acid method (DUBOIS *et al.*, 1956) after hydrolysis for 3 h in 2N H_2SO_4 in a boiling water bath. A blank tube was prepared for each hydrolysate using the same amount of sample solution but substituting 1 ml water for the 1 ml of 5% phenol.

Individual sugars were determined in these hydrolysates by paper chromatography (GAILLARD, 1965). Total uronic acids in the hydrolysates were determined by the carbazole method (DISCHE, 1947).

Lignin analysis in A, B and C fractions

It was hoped to determine this value by weighing the precipitate after hydrolyzing the sample in 2 N H_2SO_4 for 3 h. However, much lignin was soluble in the acid under the conditions used and results were reproducible with only a few samples. Instead the methoxyl content of each sample was determined by the Zeisel procedure as mentioned previously (GORDON, 1975) and the lignin content of each sample calculated, assuming that the pure lignin in each fraction contained 17.1% (wheatstraw) and 7.9% (lucerne) methoxyl. These values were determined directly by methoxyl analysis of acid-hydrolyzed total A and soluble A for wheatstraw and soluble A for lucerne. The reliability of these estimates was confirmed by measuring the extinction coefficients at 280 nm of alkaline solutions of the fractions and also the number of groups reacting with N, 2, 6-trichloro-*p*-benzoquinoneimine (GORDON, 1975). The relationship of these parameters with the methoxyl content is shown in Table 1. With the lucerne, part of the extinction coefficients may have been due to the high protein content.

Sephadex chromatography

A Pharmacia chromatographic column (K 26/40; 2.6 × 40 cm) packed with Sephadex G-50 or G-100 in aqueous ammonium acetate (.025 M) was used for the gel filtration or the B and C fractions. About 30 mg of sample was dissolved in 10 ml of the ammonium acetate and the solution passed through the column by upward elution at a flow rate of 15–20 ml/h. If the sample failed to dissolve easily it was firstly dissolved in N NaOH (2–5 ml) a nd neutralized with acetic acid (50%) before applying to the column.

TABLE 1. The relationship between the percent methoxyl groups (X) and values for the extinction coefficients in alkali at 280 nm (E 1% 1 cm; Y_1) and the number of groups reacting with N, 2,6-trichloro-P-benzoquinoneimine (guaiacol equivalent; Y_2).

	Wheatstraw		Lucerne	
	X and Y_1	X and Y_2	X and Y_1	X and Y_2
r	.997	.991	.812	.969
b	11.132[a]	.208[b]	21.383[c]	.108[b]
S.D. b	.364	.013	6.878	.012
Intercept	6.378	.067	17.753	.037
Residual S.D.	4.300	.148	14.376	.026

Numbers in same line bearing dissimilar superscripts are significantly ($p < 01$) different from each other.
S.D., standard deviation; b, regression coefficient; r, correlation coefficient.

The eluate was collected in 5 ml fractions and the absorbance at 280 nm for each fraction read on a Zeiss PMQII spectrophotometer. An aliquot of each 5 ml fraction was analyzed for carbohydrate by the phenol-sulphuric acid method and the results expressed as glucose per 5 ml. Here also a blank tube was prepared for each fraction using the same amount of sample solution but substituting 1 ml of water for the 1 ml of 5% phenol. The effect of lignin interfering with the absorbance due to carbohydrate was thus eliminated.

Crude protein and ash

Crude protein was determined by the micro Kjeldahl procedure.

Ash was determined on the C fractions by incineration at 600°.

RESULTS

The cell wall composition and solubility of the hemicellulose and lignin in KOH (10%) appear in Table 2. The most striking feature is the low solubility of the lucerne lignin compared to the wheatstraw lignin. However, the lucerne hemicellulose was slightly more soluble than that from wheatstraw. The ratios of lignin : hemicellulose in the original samples were 1:2.4 (lucerne) and 1:2.7 (wheatstraw) but in the residues the respective values were 1:1.1 and 1:3.0. A likely explanation for the big change in the atio for lucerne is that much of its lignin was free of chemically linked hemicellulose.

The yields and composition of the hemicellulose plus lignin fractions are given in Table 3. There was much protein contamination of the A and C fractions with the lucerne owing to the high crude protein content of the original material (20.6% of the

Table 2. Cell wall composition of plant material before and after extraction with 10% KOH at room temperature (% dry matter).

	Lucerne			Wheatstraw		
	Original	Residue	% dissolved	Original	Residue	% dissolved
Hemicellulose:						
total sugars	21.3	14.4	70.8	30.0	20.3	60.1
anhydro uronic acids	5.0	5.2	55.1	1.3	1.3	41.0
Total	26.3	19.6	67.7	31.3	21.6	59.3
Lignin	11.0	17.8	30.3	11.6	7.2	63.2
Residue after 10% KOH extraction	43.2	–		59.0	–	

Table 3. Yields and composition of different fractions obtained by alkali extraction of lucerne and wheatstraw (%)

	Yld (% dm)	CP	CHO	Ash	Ome	% lignin in fraction[b]
			Lucerne			
Total A + lignin	6.7	52.8	14.6	–	1.9	24
Soluble A	(43)[a]	51.5	3.3	–	2.6	33
Insoluble A	(54)[a]	48.0	18.8	–	2.0	25
Total B + lignin	7.9	9.0	55.9	–	0.6	8
Branched B	(46)[a]		39.8	–	0.4	5
Linear B	(56)[a]		47.1	–	0.6	8
Total C + lignin	9.2	50.1	13.1	8.9	1.1	14
			Wheatstraw			
Total A + lignin	24.8	2.1	66.5	–	4.3	25
Soluble A	(12)[a]		23.9	–	14.7	86
Insoluble A	(59)[a]		82.1	–	3.3	19
Total B + lignin	6.3	3.3	84.8	–	1.6	9
Branched B	(26)[a]		90.3	–	0.8	5
Linear B	(69)[a]		90.8	–	1.7	10
Total C + lignin	2.6	7.5	16.8	21.3	6.6	39

[a] Percent obtained from the original total A + lignin or B + lignin.

[b] Calculated from the methoxyl content assuming the lucerne alkali lignin contained 7.9% methoxyl and the wheatstraw alkali lignin contained 17.1% methoxyl.

CP, crude protein; CHO, carbohydrate by phenol-sulphuric acid method; OMe, methoxyl content.

dry matter). The wheatstraw contained only 2.2% crude protein so protein contamination of the extracted fractions was negligible. The carbohydrate content was measured as 'glucose' by the phenolsulphuric acid method but due to different response factors by different sugars is only a rough guide to the true content of carbohydrate.

Of the three major fractions (A, B and C) the B contained the least lignin while the C contained surprisingly large amounts. Most of the lignin recovered was found in the A fractions.

Most of the carbohydrate in the total A could be separated from the lignin by dissolving the sample in alkali and precipitating the carbohydrate with ethanol. However, even the ethanol soluble fraction did contain some carbohydrate (more with the wheatstraw than the lucerne) which must have been tightly bound to the lignin to remain soluble in the ethanol' The ratio of the neutral sugars in the lucerne soluble A was as follows:
23% galactose, 30% glucose, 26% arabinose, 21% xylose and the uronic acid content 0.3% of the dry matter. In the ethanol insoluble A the ratio of neutral sugars was 3% galactose, 31% glucose, 16% arabinose, 50% xylose and the uronic acid content 1.6% of the dry matter. For the wheatstraw soluble A the ratio was 3% galactose, 5% glucose, 24% arabinose, 69% xylose and the uronic acid content 0.4% of the dry matter. The wheatstraw insoluble A carbohydrate had the same composition as the total

Table 4. The influence of lignin on the sugar ratios and amount of uronic acid in the hemicellulose A and B fractions of lucerne and wheatstraw.

	Wheatstraw					Lucerne				
	Gal	Glu	Ara	Xyl	U.A.[c]	Gal	Glu	Ara	Xyl	U.A.[c]
A-lignin[a]	0.0	0.0	5.9	94.1	2.1	0.0	0.0	0.0	100.0	6.6
A-lignin[b]	0.0	0.0	5.2	94.8	4.4	0.0	0.0	0.0	100.0	7.2
A + lignin	0.3	3.6	7.1	89.0	2.4	12.5	29.8	22.0	35.7	1.3
LB-lignin[a]	0.0	12.0	9.8	78.2	0.3	0.0	11.4	7.5	81.1	1.5
LB-lignin[b]	2.9	12.5	10.1	74.5	8.7					
LB + lignin	0.6	8.2	11.8	79.4	8.1	3.5	31.9	15.3	49.3	5.0
BB-Lignin[a]	10.6	0.0	28.8	60.6	7.9	40.0	12.0	44.0	4.0	22.3
Bb-lignin[b]	4.7	3.1	30.4	61.8	12.7					
BB + lignin[c]	4.7	11.0	27.0	57.2	8.6	18.6	18.6	35.0	27.8	7.7

LB, Linear B; BB, Branched B.

[a] Sugar ratios for fractions obtained from delignified samples reported by Gaillard. No correction was made for xylose present in aldobiuronic acids.

[b] Sugar ratios for fractions obtained from delignified samples in this experiment. No correction was made for xylose present in aldobiuronic acids.

[c] U.A., uronic acid expressed as a percentage of the sample dry matter. No correction was made to the xylose value for any xylose attached to uronic acid.

A. The carbohydrate associated with the soluble A lignin was similar to the branched B polymers in having more arabinose and less xylose.

When the total B fractions were separated by iodine precipitation into linear and branched fractions, most of the lignin was precipitated with the linear polymer.

The influence of the lignin on the sugar ratios in each fraction can be seen in Table 4. The values for samples from delignified plant material were recalculated from the paper by GAILLARD (1965). The uronic acids are expressed as a percentage of the sample dry matter and the sugars as a percentage of their sum. GAILLARD's results were checked by repeating the analysis for fractions from a sample of delignified wheatstraw and lucerne. The carbazole method of uronic acids analysis gave higher results than the method used by GAILLARD but otherwise her results were confirmed. Glucose and arabinose are likely to be higher in the samples containing lignin because any glucan (starch?) is probably dissolved and arabinofuranosides lost during delignification in slightly acid solution at 80°.

With the wheatstraw A and linear B the presence of lignin had very little effect on the sugar ratios, although there was a small amount of galactose present in the linear B which also had an excessive amount of uronic acid. However, the last point was true of the fractions obtained from delignified samples analysed in this work.

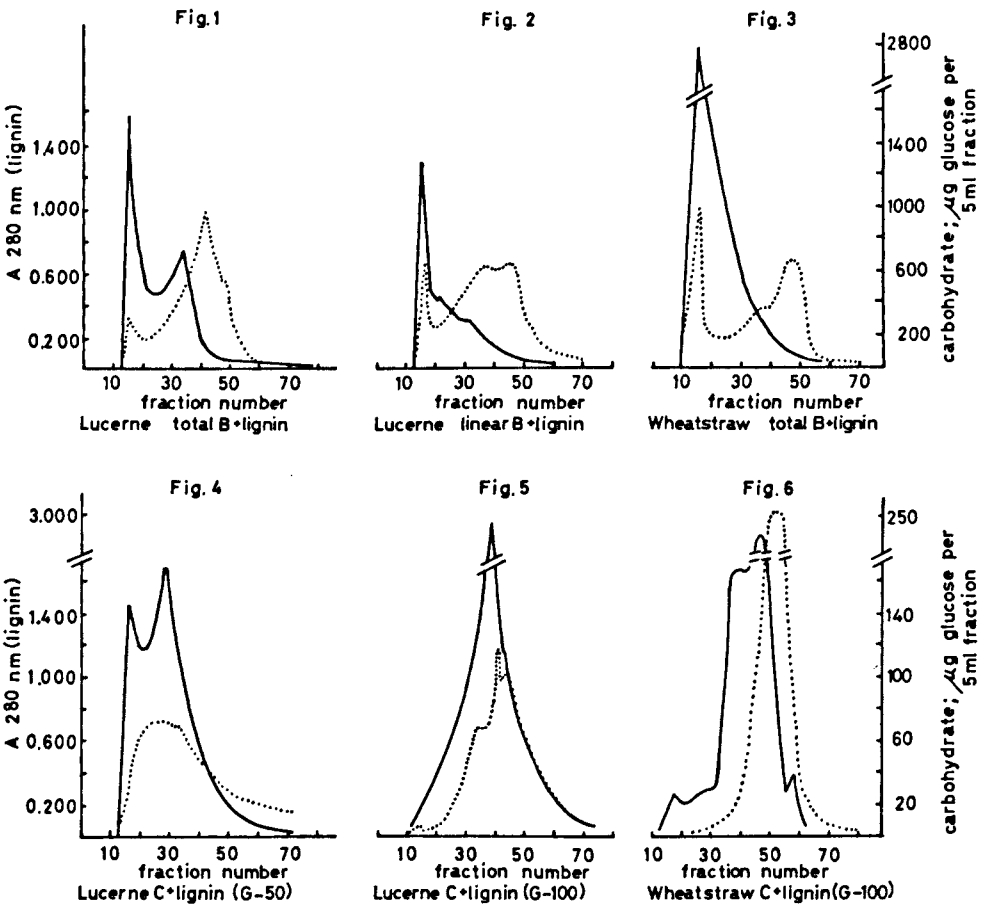
With the lucerne, the lignin probably interfered with the separation of the A, B and C fractions since there is obviously major contamination of one fraction with another. The lignin may link one hemicellulose fraction with another.

Gel-filtration was performed on the total B, linear B, branched B and C fractions. The elution profile of lucerne total B plus lignin on Sephadex G-100 is shown in Figure 1. Since the second and major peak (Kav 1.0) of the lignin (as measured by the absorbance at 280 nm) did not coincide with the second carbohydrate peak (Kav 0.7) it appears that most (e.g. 90%) of the carbohydrate in the hemicellulose B fraction is not linked covalently with lignin. The first carbohydrate peak (Kav 0.0) in Figure 1 is probably due to the linear hemicellulose B (with possible some hemicellulose A) polymer since its elution profile shows only this one peak at the void volume (Figure 2). The profile for the branched B was almost identical with that from the total B so that it was probably contaminated with linear B. Attachment of lignin to the linear carbohydrate probably prevents its precipitation with iodine.

With the wheatstraw total B there was only one carbohydrate peak (Kav 0.0) upon filtration through Sephadex G-100 (Figure 3). This is in sharp contrast to the two carbohydrate peaks (Kav 0.0 and 0.7) found for lucerne. The linear and branched B fractions gave similar profiles to the total B.

With all the B fractions, from both lucerne and wheatstraw, there was a small lignin peak and a large carbohydrate peak in the void volume. These possibly represented a lignin-carbohydrate complex, covalently linked.

The linear fracrions from both plants contained a greater proportion of the total lignin in this peak than their corresponding branched fractions (table 5). Likewise all three wheatstraw fractions had more lignin in this peak than the three lucerne frac-



Figures 1, 2 and 3. Gel-filtration profiles on Sephadex G-100 of lucerne and wheatstraw hemicellulose B fractions which also contained lignin.

Figures 4, 5 and 6. Gel-filtration profiles on Sephadex G-50 (fig. 4) and G-100 of lucerne and wheatstraw hemicellulose C fractions which also contain lignin.

(—— carbohydrate; 4g glucose/5 ml; .... lignin, A280 nm).

tions. Both linear fractions contained more lignin relative to carbohydrate than the two branched fractions. Of the total areas under the absorbance curve at 280 nm, 16% and 11% respectively were due to the peak at V_o with the lucerne linear and branched B polymers. For wheatstraw the values were 46% and 37%. Thus there is a greater frequency of lignin-carbohydrate bonds in wheatstraw B hemicellulose than lucerne and with both plants most of these occur in the linear polymers.

Two carbohydrate peaks were clearly apparent when the lucerne C was passed through Sephadex G-50 (Figure 4). The first peak contained mainly arabinose with only traces of the other sugars. It may have been largely due to pectic substances. The second peak was more hemicellulosic in nature, containing 10% galactose, 24% glucose, 48%

Table 5. Areas (per mg of total lignin applied to column) of lignin peaks at V_0 and the ratios of lignin to carbohydrate at V_0 for lucerne and wheatstraw hemicellulose B polymers.

	Area (mm^2) V_0 lignin peak/mg lignin	A280 × 10^4 / βg glucose
	Lucerne	
Total B	145	2.1
Linear B	239	4.7
Branched B	136	1.6
	Wheatstraw	
Total B	263	3.6
Linear B	436	5.9
Branched B	256	1.5

arabinose and 20% xylose. Traces of rhamnose and aldobiuronic acids were also present. There was no separation of components absorbing at 280 nm.

On G-100 the lucerne C gave only one carbohydrate peak but four lignin peaks (Kav 0.0, 0.8, 1.1 and 1.3), some of which may have been due to protein (Figure 5). The wheatstraw C was not passed through Sephadex G-50 but with G-100 the lignin was adsorbed and only one sharp peak at Kav 1.3 was seen (Figure 6). Pure glucose, when applied to the column had a Kav of 1.0 and so the adsorption of the second carbohydrate peak (Kav 1.1) was probably caused through its linkage to the adsorbed lignin.

Discussion

In a discussion of lignin-carbohydrate linkages Lai and Sarkanen (1971) consider four possible types of linkages, namely:

1. benzyl-ether links which should be readily hydrolyzable by acids but more resistant towards bases.
2. ester linkages including those linking glucuronoxylans to lignin. Such linkages should be more frequent among legume hemicelluloses (especially the branched B polymers) which contain more uronic acids than grasses (Gaillard, 1965). Ester bonds are known to link cinnamic acids to lignin in grasses (Gordon and Neudorffer, 1973).
3. chemically stable links such as carbon-carbon bonds and carbon-oxygen bonds (excluding benzyl ether bonds).
4. phenyl-glycosidic bonds which are subject to hydrolysis by acids and enzymes.

In the complexes studied in this work, types 1, 3 and 4 are likely to be present but it is not possible to deduce which type predominates.

The finding that the lignin in wheatstraw has a higher frequency of linkages with carbohydrate than the lignin from lucerne is in agreement with the suggestion (Gor-

DON, 1975) that the lignin in legumes has a smaller effect on the digestibility of hemicellulose than does the lignin from wheatstraw despite the fact that there is more lignin per gramme of hemicellulose in the former. Alkali lignins from lucerne appear to be more highly condensed than those from grasses and have fewer chemical sites available for carbohydrate attachment (GORDON, 1975).

The lucerne lignin had a greater effect on the separation of the hemicellulose than wheatstraw lignin which suggests either that its lignin acted as a link between the different hemicellulose fractions or that it prevented portions of each fraction from being dissolved in alkali. The former possibility appeared the more likely as the lucerne hemicellulose was more soluble in the 10% KOH than the wheatstraw hemicellulose. The highly condensed lucerne lignin might link the different hemicellulose fractions by entanglement or by actual covalent links.

There is some evidence for the involvement of a particular carbohydrate polymer high in arabinose being linked to the lignin of fraction A with both plants.

Since significant amounts of lignin can be soluble in ethanol even under neutral or acidic conditions the finding that more lignin appeared in the C fraction than the B fraction is not particularly surprising. However, that 37% of the total lucerne lignin recovered was found in this fraction compared with only 46% for the A fraction is quite high considering the original insolubility of the lucerne lignin relative to the wheatstraw lignin where the corresponding values were 14% and 78%. The lignin present in the wheatstraw C fraction appears to be of lower molecular weight material than that from lucerne.

The low methoxyl content of the lucerne lignin (7.9%) is in good agreement with other work for lignin extracted from lucerne with alkali. This lignin is not typical of the total lignin in the plant since lignin isolated by other methods contains a higher content of methoxyl groups (GORDON, 1975).

Lignin-carbohydrate complexes extracted from ballmilled samples of a grass hay (mainly *Bromus inermis*) and lucerne (A. J. GORDON, unpublished results) with a neutral solvent (dioxane: water, 9:1) differed from these samples in containing very high proportions of glucose (41–44%) and significant proportions of rhamnose (7–16%). Such complexes should retain linkage type 2 in addition to types 1, 3 and 4. MORRISON (1974) found even higher proportions of glucose (> 50%) but no rhamnose in complexes extracted from *Lolium perenne* with dimethylsulphoxide. He suggested that the glucose was most likely due to cellulose. On the other hand, complexes extracted from ball-milled cornstalks with dioxane-water contained less glucose (18–28%) and no rhamnose (A. J. GORDON, unpublished results). The excess amounts of glucose in the lucerne compared with the wheatstraw fractions in the present work are probably due to extraction of both starch and glucan present in the linear B hemicellulose (GAILLARD, 1965).

In MORRISON's (1974) complex the linear and branched polymers contained equal amounts of lignin in contrast to the results of this work.

The gel-filtration elution profiles of the B fractions from both plants were similar

to those reported for the A fractions of cornstalks (GORDON, 1975) in that a minor proportion of the lignin and most, if not all, of the carbohydrate were eluted together in the void volume. The major differences were that the A fractions were chromatographed on Sephadex G-200 and a smaller proportion of their total lignin was associated with carbohydrate in the void volume.

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FUNGAL CELLULASES AND HEMICELLULASES AND THEIR APPLICATION TO THE ANALYSIS OF FORAGE

D. I. H. JONES

Welsh Plant Breeding Station, Aberystwyth, United Kingdom

SUMMARY

The main characteristics of fungal cellulases and hemicellulases, methods of preparation and mode of action, are briefly reviewed in relation to the potential application of these enzymes to the analysis of forage. Some current applications of fungal carbohydrases in analysis are discussed, particularly their use for predicting forage digestibility.
Key Words: Cellulase, hemicellulase, forage analysis, digestibility.

INTRODUCTION

The microbial degradation of cellulose and its associated polysaccharides is a vital process in the maintenance of the carbon cycle in nature. The process results in the decay of vegetation, the recycling of nutrients through the soil for new plant growth and the release of billions of tons per annum of carbon dioxide into the atmosphere. Microbial degradation is also important in other respects. For example, symbiotic association of carbohydrases in the gut of herbivores enables these animals to subsist and produce on diets largely composed of cellulose and hemicelluloses, and vast areas of natural and improved grasslands are thereby utilized for the production of human food. Not all the activities of cellulolytic organisms are as welcome to man. Destruction of timber by wood rotting fungi and damage to cotton and other textiles, paper and paper products, etc., represents a considerable annual financial loss in most areas of the world. Other cellulolytic fungi are phytopathogenic and are responsible for many of the economically important diseases of food crops, (WOOD, 1960).

Several comprehensive reviews of the biological degradation of cellulose have been published (SIU, 1951; GASCOIGNE and GASCOIGNE, 1960; NORKRANS, 1963; NORKRANS, 1967; JURASEK, COLVIN and WHITAKER, 1967; WHITAKER, 1971; PATHAK and GHOSE, 1973). It is not the intention in the present paper to restate or supplement the content of these reviews; the aim is to assess the characteristics of fungal cellulases and hemicellulases pertinent to their use in the laboratory assessment of the nutritive value of forage crops.

DISTRIBUTION

Since cellulose and its associated polysaccharides are widely found in nature it is not surprising that fungi able to utilize these substrates are equally widely distributed. They are abundantly distributed in soil (PUGH, 1974), where they are vital for the decay of plant residues, on cotton and cotton products, decaying timber, paper and paper products, etc. REESE and MANDELS (1962) have listed the important fungi involved in the deterioration of cotton. Most belong to the Fungi Imperfecti, the most common species studied being *Chaetomium globosum*, various species of *Aspergillus* and *Fusarium*, *Trichoderma viride* (and *koningii*), and *Myrothecium verrucaria*. The primary attack on lignified cellulose, forest litter and wood is by Basidiomycetes, some 2000 species having been classified as wood destroying fungi (NORKRANS, 1963). A notable feature of some wood rotting fungi (the white rots) is their ability to decompose lignin as well as cellulose and hemicelluloses.

Most, if not all, cellulolytic fungi also contain pentosanases or hemicellulases. These have been studied in less detail than the cellulases, but the common cellulolytic fungi found on cotton have all been shown to contain hemicellulases (GASCOIGNE and GASCOIGNE, 1960).

PREPARATION OF ENZYMES

The cellulolytic and associated enzymes of fungi are found in the culture liquid in which the organism is grown. Cellulase is generally assumed to be an inducible enzyme formed only in the presence of cellulose, but there is evidence (REESE and MANDELS, 1962) that the true inducer is cellobiose, not cellulose. Culture solutions therefore comprise a mixture of salts, nutrients and cellulose. After the requisite growth period, the cells and residual cellulose are removed by centrifugation of filtration and the cell free solution utilized directly or freeze-dried. The activity of the preparation thus obtained is greatly influenced by a variety of factors, e.g. species and strain of organism, source of C and N, mineral supply, pH, aeration of culture fluid, etc.

Suitable conditions for the production of *Trichoderma viride* cellulase have been described by REESE and MANDELS (1963). The culture solution comprises (per litre) KH_2PO_4 (2.0 g), $(NH_4)_2SO_4$ (1.4 g), urea (0.3 g), $MgSO_4\ 7H_2O$ (5 mg), $CoCl_2$ (2 mg), proteose peptone (10 g) and 'Solka Floc' (10 g). The medium (100 ml in 500 ml conical flasks) is sterilized, inoculated with *T. viride* spores from a potato dextrose slant, incubated at 30 °C with continuous shaking for 7–10 days, and the culture fluid removed by filtration or centrifugation. The fluid may then be freeze-dried or an acetone powder prepared. Using this procedure, crude cellulase and hemicellulase preparations of high activity on forage, forage cell wall and forage cellulose and hemicellulose preparations can be obtained (BAILEY and JONES, 1971). Some factors influencing the yield and activity of fungal cellulases have been discussed by MANDELS and WEBER (1969). In their comparison of several fungi, many of the enzymes tested showed a higher ac-

tivity than *T. viride* cellulase against soluble cellulose substrates such as carboxymethyl cellulose, but the most reliable producer of a cellulase active against insoluble cellulose substrates was *Trichoderma viride*. The cellulase from this fungus was found to produce over 50% weight loss in cotton slivers in 35 days, some 30% more than from the best of the other cellulases tested. It has been shown more recently that the yield of cellulase and other carbohydrases can be increased by the addition of surfactants to the culture solutions (REESE and MAGUIRE, 1969), and a mutant strain of *T. viride* obtained by irradiation was found to secrete twice the cellulase of the parent strain (MANDELS, WEBER and PARIZEK, 1971).

In general, all crude cellulase preparations also show hemicellulase, or at least xylanase, activity but little specific attention appears to have been given to the production of hemicellulases. Xylanase may be obtained free of cellulase activity by growing fungi on xylan rather than cellulose substrates (REESE and MANDELS, 1959). Obtaining cellulases free of xylanases appears, however, to be difficult, and many workers have found that even purified cellulase components retain xylanase activity.

Large scale production of cellulases is largely confined to Japan, using the submerged culture or Koji-type processes. The cultures are grown in aerated submerged fermentation vessels on nutrients with wheat bran, soyabean cake, filter paper, straw, sawdust, etc., or on steamed wheat bran spread on trays. In each case the culture solution is separated off after the growth period and the crude enzyme precipitated with ammonium sulphate (PATHAK and GHOSE, 1973). Many highly active cellulases are now commercially available for laboratory use; those derived from *T. viride* have generally been found more active on forage substrates (HARTLEY, JONES and WOOD, 1973; JONES and HAYWARD, 1975).

The purification of crude cellulase preparations is complicated by the multi-component nature of the cellulase complex (see below), and purification therefore results in the resolution or partial resolution of the various components. All the usual techniques of protein separation have been employed in attempts to purify the complex and these have been reviewed by GASCOIGNE and GASCOIGNE (1960), HASHIMOTO and NISIZAWA (1963) and others. The earlier techniques have now been largely superseded, particularly as a result of developments in gel filtration and ion exchange media. Detailed descriptions of the technique used to obtain highly purified components of the cellulase complex are found by reference to SELBY and MAITLAND (1967); SELBY (1969); ERIKSSON (1969); WOOD and MCCRAE (1972); HALLIWELL and GRIFFIN (1973). Techniques suitable for the separation of hemicellulases and their purification have been described recently by DEKKER and RICHARDS (1975).

NATURE OF THE CELLULASE COMPLEX AND ITS MODE OF ACTION

It has long been known that although many organisms can achieve extensive solubilisation of native cotton cellulose, cell free extracts of these organisms have little effect on such a substrate although well able to hydrolyse modified celluloses. It is clear that

some enzyme or factor present in the growing organisms is missing from the extracellular extract and that this enzyme or factor is necessary for attack on highly ordered forms of cellulose. To account for this observation, REESE, SIU and LEVINSON (1950) suggested that at least two enzymes were involved in the solubilization of cotton, viz. a C_1 enzyme said to produce linear anhydroglucose chains from cotton cellulose and a C_x enzyme able to depolymerise these chains, thus permitting their final hydrolysis to glucose by cellobiase, The C_x enzyme was also considered to be directly effective on celluloses modified by swelling, reprecipitation, etc.

The isolation and characterization of the C_1 components has, however, proved an elusive problem despite considerable evidence accruing to indicate the validity of the C_1-C_x concept. The nature of the C_1 action was regarded by some as a hydrolase, by others as having an undefined effect in modifying crystalline cellulose and enabling attack by the C_x enzyme. Other factors were also implicated; MARSH *et al.* (1953), noted certain changes in cotton during the initial stages of attack by fungi before any loss in weight or tensile strength could be noted and suggested the participation of a swelling factor. Progress was considerably hampered by the failure to obtain significant degradation of native cotton by cell-free extracts, i.e. extracts containing appreciable C_1 activity. SELBY *et al.* (1963), however, found that filtrates from *Myrothecium verrucaria*, suitably manipulated, could achieve 30% solubilisation of processed cotton. Subsequently, even greater solubilisation of native cotton was achieved in cell-free preparations of *Trichoderma viride* and *T. koningii* (MANDELS and REESE, 1964; LI *et al.*, 1965; HALLIWELL, 1965).

Extracts containing the C_1 component thus became available for study and provided a stimulus for its isolation and characterization. In their work, MANDELS and REESE had isolated a new protein believed to be the C_1 component, together with C_x (or carboxymethyl cellulase) and cellobiase. Attempts to purify these components further results in each of the components retaining some of the activity of the other components and a strong synergistic effect was apparent, viz. C_1 had little effect in solubilizing celulose except in the presence of C_x (SELBY and MAITLAND, 1967; WOOD, 1968, 1969). In the course of this and other work it was apparent that the C_x enzyme is itself multi-component. KING and VESSAL (1969) summarized the nature of the cellulase complex as comprising:-

(a) a C_1 enzyme regarded by some workers as a hydrolase yielding cellobiose from crystalline cellulose and by others as having an unspecified modifying effect on crystalline cellulose, but in any event necessary for attack on highly orientated forms of cellulose;
(b) the C_x enzymes, β 1.4 glucanases comprising both exo- and endoglucanases, i.e. glucanases removing single terminal units from the cellulose chain and those acting at random on the chain; the activity of these C_x or CM cellulases is usually measured by their action on carboxymethyl cellulose;
(c) various β-glucosidases able to hydrolyse all the β-dimers of glucose, generally referred to as 'cellobiase'.

In recent years several workers have used improved separation procedures to isolate purified components of the *Trichoderma* cellulase complex, and have examined the inter-relationships of the components in achieving solubilization and hydrolysis of highly ordered forms of cellulose. For example, HALLIWELL and RIAZ (1971), resolved the complex into four components, namely, cellobiase, a C_1 component, (cellobiohydrolase), and two new components, viz. a CM-cellulase and a C_2 component. These new components together form the composite CM-cellulase-short-fibre forming activity, the latter being an ability to produce short fibres from cellulose. All four components were necessary for the efficient solubilization of cotton cellulose. WOOD and MCCRAE (1972) found that a purified C_1 readily degraded phosphoric acid treated cellulose to yield cellobiose; dewaxed cotton was degraded to a far lesser extent, again to yield cellobiose. They concluded that the C_1 component was a β-1, 4-glucan cellobiohydrolase which acts synergistically with a C_x component to solubilize native cellulose. HALLIWELL and GRIFFIN (1973) obtained a purified component free from the associated activities of the cellulase complex and which hydrolysed both simple and complex forms of cellulose yielding cellobiose. Their evidence suggested synergism of the C_1 component with cellobiase rather than with a C_x component. A C_1 component free from CM-cellulase or β-glucosidase has also been isolated by BERGHEM and PETTERSSON (1973). They concluded that the enzyme was a glycoprotein and a β 1,4 glucan cellobiohydrolase, and the purified enzyme represented 0.14% of the commercial cellulase used for the fractionation.

Few workers appear to have studied the effect of components of the cellulase complex on forage cell wall substrates. HARTLEY *et al.*, (1973), however, showed that purified C_x and a C_x fraction containing β-glucosidases from *Trichoderma koningii* were highly active on grass cell walls though not very active on cotton. Purified C_1 showed low activity on both substrates while C_1–C_x synergism was less marked on grass cell wall than on the cotton substrate. Purified enzymes from *Fusarium solani* differed markedly in their behaviour compared with *T. koningii*. The C_x component of *F. solani*, for example, had little effect on either cotton or cell wall substrates.

The limited evidence available on the mode of action of hemicellulases has been reviewed by GASCOIGNE and GASCOIGNE (1960). A multienzyme complex is again suggested, the random acting components yielding polyxylosans which are further hydrolysed by other components to xylose. DEKKER and RICHARDS (1975) have recently described the purification, properties and mode of action of a hemicellulase from a fungal plant pathogen producing five extra-cellular hemicellulases.

ASSAY AND GENERAL CHARACTERISTICS

It is clear from the foregoing discussion of the nature of the cellulase complex that only assays based on highly orientated forms of cellulose can be regarded as measuring the activity of the complex as a whole, i.e. the enzyme system capable of converting

crystalline, amorphous or modified forms of cellulose quantitatively to glucose. The method of assay may then take the form of (i) measurement of loss in weight of substrate, (ii) increase in viscosity or, (iii) measurement of reducing sugars, glucose, xylose, etc. produced. Since soluble products of longer chain length than glucose or xylose may be formed, the measurement of reducing sugars may be misleading, and HALLIWELL (1963) suggests the method of choice to be an estimation of loss in weight from gravimetric or chemical determination of residual insoluble substrate. It is unfortunate that many commercial cellulases are still assayed by the manufacturers or suppliers in terms of their activity on carboxymethyl substrates, an activity which has little discernible relationship to the ability of the enzyme to solubilize forage cell wall or insoluble celluloses (HARTLEY *et al.*, 1973; JONES and HAYWARD, 1975).

The variety of substrates, assay procedures and assay conditions used by different workers undoubtedly contribute to the conflicting results quoted in the literature regarding the characteristics of cellulases. The pertinent literature has been reviewed by GASCOIGNE and GASCOIGNE (1960) and MANDELS and REESE (1963). In general, pH optima in the region of 4.5 have been found with an active range of 4–7, but the enzymes from certain fungi, e.g. *Myrothecium verrucaria* appear to have a higher optimum. The nature of the substrate influences the optimum pH, a higher optimum being found for *Trichoderma koningii* on cellulose as opposed to carboxymethyl cellulose substrates. Temperature optima have proved more difficult to define since two factors are involved, viz. the increase in reaction velocity and denaturation of the enzyme. Although high temperatures may thus give a higher initial rate, a greater amount of the substrate may be hydrolysed at lower temperatures. Fungal cellulases have generally been found to be inactivated by comparatively short periods of heating at 70 °C. Temperature optima of about 50–60 °C have often been quoted, but most workers currently favour an incubation temperature of 37°–40 °C.

The literature regarding the effects of metal ions is particularly conflicting. Some workers have found that Cu^{2+} and Mn^{2+} inhibit and others that they stimulate the activity of *Trichoderma* cellulase. From the reviews quoted above it appears that the ions of Hg, Ag, Cu, Cr, Pb and Fe inhibit cellulase activity while those of Mg, Co, Ca and phosphate stimulated activity. HALLIWELL and GRIFFIN (1973) have recently shown their purified C_1 component from *Trichoderma* to be inhibited by Cu, Zn and Fe ions. The same enzyme was also inhibited by its product, cellobiose, a saccharide generally found in earlier work to be inhibitory to less purified cellulases. Proteins have been reported to stimulate cellulase action, possibly due their effect in protecting the enzyme from denaturation. Toluene, antiseptics and substituted phenols have all been shown to be inhibitory but antibiotics and phenol itself are apparently not (GASCOIGNE and GASCOIGNE, 1960). A variety of other substances have been assessed as potential inhibitors, including the natural cellulase inhibitors found in many plants. These appear to be polyphenols which form physical complexes which may be broken by the addition of another protein. MANDELS and REESE (1963) suggest that they may be produced by the plant to protect itself against fungal attack. In response, some

fungi have developed hydrolytic enzymes relatively resistant to some of the natural inhibitors, e.g. virulent fungi attacking plants high in polyphenols may therefore contain polyphenol oxidases or produce an enzyme not inhibited by polyphenols.

A factor which clearly influences cellulase activity is the nature of the substrate. COWLING and BROWN (1969) have discussed in detail the influence of fibre structure on its susceptibility to cellulase. Any structural feature of the substrate which limits its accessibility to the cellulase will diminish susceptibility; highly crystalline forms of cellulose with tightly packed chains are thus less susceptible. Treatment with alkali or acid causes swelling of the fibres, thus permitting greater access. Of particular importance in relation to forage analysis is the physical protection of cellulose or hemicellulose by encrusting substances, notably lignin. Removal of wood lignin, e.g. by the chlorite process, yields a product very susceptible to hydrolysis. Similarly, the removal of forage lignin (BAILEY and JONES, 1971) results in forage cell wall cellulose being completely hydrolysable by cellulase. The chemical treatments involved in removing lignin however, may have effects on the cell wall polysaccharides which could influence their susceptibility to attack.

Very little information appears to be available on the characteristics of fungal hemicellulases. The limited data indicates inhibition by the same metal ions as for cellulases, but the pH optima are often higher and temperature optima lower than for cellulases. The properties of a highly purified hemicellulase from *Caratocystis paradona* have been detailed by DEKKER and RICHARDS (1975) who quote an optimum pH of 5.5 and a temperature optimum of 40 °C.

APPLICATIONS TO THE ANALYSES OF FORAGE

The ruminant derives a considerable proportion of its dietary energy from the digestion of cell wall polysaccharides when fed on forage diets. These polysaccharides comprise fibres of organised, crystalline cellulose in a matrix of hemicelluloses which are associated physically and chemically with lignin. The digestibility of the structural polysaccharides is greatly influenced by the amount and distribution of this lignin component, and the digestibility of the cell wall constituents is thus much reduced in mature highly lignified tissue. The cell wall constituents also influence the voluntary feed consumption of ruminants fed on roughages. Intake appears to be primarily influenced by the rate of passage of digesta through the gut, a rate determined by the speed with which the fibrous portion of the feed is reduced to a fine particle size as a result of microbial and physical action in the rumen. These aspects of forage quality have been the subject of several reviews, including those of VAN SOEST (1969); RAYMOND (1969) and JARRIGE *et al.*, (1973).

The estimation of digestibility is of crucial importance in the evaluation of quality in forages and, in consequence, considerable efforts have been made to develop laboratory techniques which enable *in vivo* digestibility to be predicted. Much of the earlier

work centred on prediction from chemical composition, notably of the fibre content or of particular cell wall components (GAILLARD, 1962; SULLIVAN, 1964). The development of improved rumen fermentation techniques greatly increased the precision of laboratory estimations of digestibility, particularly the two stage rumen inoculum – pepsin technique of TILLEY and TERRY (1963). Techniques using rumen inoculum have, however, inherent disadvantages, notably the variability in activity of different batches of inoculum obtained from the cannulated donor animals. The use of an active cellulase-hemicellulase system has obvious advantages in circumventing many of the problems associated with using fresh rumen inoculum. Ideally, the enzyme system should be derived from rumen microbes. Cell-free extracts from disrupted mixed rumen bacteria rapidly hydrolyse plant hemicelluloses (GAILLARD *et al.*, 1965; BAILEY and MCRAE, 1970; BEVERIDGE and RICHARDS, 1973); these extracts, however, have little or no cellulolytic activity. Attention has therefore been directed towards fungal sources of these enzymes. Although early attempts at using commercial cellulase preparations had little success, JARRIGE *et al.* (1970) reported a good correlation between the *in vivo* digestibility of forages and their solubility in a Basidiomycete cellulase solution. JONES and HAYWARD (1973) found solubility of forages in a crude *Trichoderma viride* cellulase to be well related to their *in vivo* digestibility, the crude cellulase used showing cellulase. hemicellulase and protease activity. A microtechnique involving cellulase digestion of forage cell wall material was described by HARTLEY *et al.* in 1974. They found forage *in vivo* digestibility could be predicted with high precision from the release of phenolic compounds during cellulase treatment.

Pretreatment of forage with acid pepsin was found by JONES AND HAYWARD (1975) to increase the solubility of forages in cellulase and to significantly improve the correlation of solubility with *in vivo* digestibility. Using the pepsin pretreatment, the precision of predicting *in vivo* digestibility was at least equal to, and generally better than, that attained by the two stage *in vitro* procedure. Different fungal cellulases could be used with comparable results if the forage was pepsin treated although the cellulases differed markedly in their ability to solubilize the forages in the absence of pepsin. The results suggest that pepsin treatment improves the accessibility of the cell wall polysaccharide to the fungal enzymes, a suggestion supported by electron micrographs of leaf sections treated with cellulase with and without pepsin treatment (SELIM *et al.*, 1975).

Apart from their use as simple solubilizing agents, fungal cellulase and hemicellulase preparations offer a more meaningful means of hydrolysing forage cell wall polysaccharides than is possible by chemical reagents. BAILEY and JONES (1971), for example, used a *Trichoderma viride* cellulase-hemicellulase preparation to study the rate and extent of digestion of ryegrass cell wall and polysaccharides isolated from the cell wall. A rumen hemicellulase was also used, and comparison with *in vivo* nylon bag digestion showed the enzyme results to parallel those in the rumen. Essentially the same techniques were used by JONES and BAILEY (1972 and 1974) to relate the voluntary intake of forages to their susceptibility to cellulase-hemicellulase action, and study the

effect of heat treatment on the accessibility of cell wall polysaccharides to enzymic hydrolysis. Crude enzyme preparations were used in these studies, and an interesting and potentially fruitful future development is the application of more purified enzyme systems to forage cell wall materials in order to assess the relative accessibility to enzymes of more specific substrates.

The application of fungal enzymes to studies of the nature of the ligninpolysaccharide linkages in forage cell walls is another promising development. Removal of lignin by chemical treatment results in a holocellulose completely and readily hydrolysed by *T. viride* cellulase – hemicellulase, (BAILEY and JONES, 1971). HARTLEY (1973) showed that carbohydrate esters of ferulic acid are released when grass cell wall preparations are treated with cellulase. There was evidence that these esters could be carbohydrate-lignin linkage units. The existence of such linkages could well be responsible for the marked decrease in digestibility which accompanies the increasing lignification of forages.

Fungal carbohydrases are thus finding considerable application in studies on forage cell wall polysaccharides. This is largely due to the discovery of active cellulase and hemicellulase preparations able to solublize forage cell wall substrates. It seems likely that further application of techniques using fungal carbohydrases will contribute significantly to our understanding of the fundamental structure of forage cell walls and the factors influencing their accessibility to digestion.

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DIGESTION OF CARBOHYDRATES IN THE INTESTINES OF DAIRY COWS

A. TH. VAN 'T KLOOSTER and BLANCHE D. E. GAILLARD[1]

Agricultural University, Wageningen, The Netherlands

SUMMARY

In cows fitted with cannulas into the duodenum and distal ileum, the relative apparent digestion of ethanol-soluble carbohydrates, α-glucose polymers, water-soluble carbohydrates, polysaccharides hydrolysable with 2 N H_2SO_4 ('hemicellulose') and polysaccharides hydrolysable with 72% H_2SO_4 ('cellulose') was studied. Composition of the diets varied from grass alone to rations containing 70% concentrates.

The total apparent digestion of the different carbohydrate (CH) fractions declined with the range ethanol soluble CH, α-glucose polymers, water-soluble CH, 'hemicellulose' and 'cellulose'.

Of the total apparent digestion of ethanol soluble CH 15% took place in the intestines of which 1/3 in caecum and colon.

The quantities of α-glucose polymers in duodenal contents amounted to 9 to 27% of the starch present in the rations. From 50 to 65% of these α-glucose polymers was digested in the small intestine. Fermentation of α-glycose polymers during passage through the caecum and colon resulted in an apparent disappearance amounting to about 5% of the starch intake.

When fed concentrate-rich rations about 200 to 400 g of hexoses (10 to 20% of the estimated hexose requirement) was absorbed daily from the small intestine of dairy cows.

The relative apparent digestion of the water-soluble CH was low in the small intestine but in caecum and colon it was higher than of any of the other CH fractions. Digestion of 'hemicellulose' and 'cellulose' was virtually absent in the intestines.

The relative disappearance of gross energy (GE) proximal to the duodenum, in the small intestine and in caecum and colon was 38 to 49%, 50 to 60% and 1 to 12% respectively. The GE calculated from CH apparently fermented proximal to the duodenum was 1.3 to 1.7 times as high as the apparent disappearance of GE measured at this site of the gut. This could be explained by the increase in the content of other extractable matter and of protein between intake and duodenal dry matter.

INTRODUCTION

In the forestomachs of the ruminant fermentative digestion of carbohydrates (CH) occurs on a massive scale. As a consequence the contribution of the intestines to the carbohydrate digestion is relatively small. Nevertheless, ruminants like other mammals require considerable quantities of glucose for cell metabolism (brain, nerve, muscle) and synthesis of lactose and fat. The overall rate of glucose metabolism in the

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body has been estimated by the rate of dilution of radioactive glucose in the blood. It seems reasonable to assume that in the dynamic steady state this rate equals the rate of glucose utilization and that after a long enough period of fasting the glucose turnover approaches the glucose requirement. BALLARD, HANSON and KRONFELD (1969) computed a regression equation ($\log G = 0.456 + 0.807 \log W$) relating glucose entry rate (G-mg/min) and bodyweight (W). This applied to sheep, goats and cattle fasted for 24 to 96 h. The data were taken from different experiments in which the modes of tracer administration used included single injection, primed infusion and continuous infusion. From this regression equation a glucose entry rate of 650 g glucose per day could be calculated for a 550 kg ruminant and of 94 g per day for a 50 kg sheep. ARMSTRONG and BEEVER (1969) mention an estimate of 4.4 g glucose per $kgW^{0.75}$ or about 82 g per day for a sheep weighing 50 kg. This agrees with the estimate of 4.75 g glucose per $kgW^{0.75}$ calculated by BOEKHOLT (thesis, in preparation) from glucose entry rates of sheep fasted for more than 24 hours.

As glucose is the precursor of lactose, about 50 g of glucose will be used for the synthesis of 1 kg of milk. Glucose is further needed for the glycerol incorporated in milk fat and for milk fat synthesis (BAUMAN and DAVIS, 1975).

BOEKHOLT calculated the glucose requirement for glycerol synthesis as 4.8 g per kg fat corrected milk (FCM) and for the production of 50% of the NADPH needed in fatty acid synthesis as 14.6 g per kg FCM. This would mean a total requirement of 70 g glucose per kg milk or a requirement of 1225 to 2625 g absorbed glucose per day for a cow producing 10 to 30 kg FCM.

The most efficient way of obtaining the required glucose would be a straight absorption from the gut. Absorption of glucose from the forestomachs is negligible on ordinary rations as the microbiological fermentation results in the production of VFA from CH. Glucose, fructose and sucrose are rapidly metabolized by rumen bacteria (SUTTON, 1968) and free glucose levels in rumen fluid are hardly detectable (HUNGATE, 1966; BAILEY, 1967). According to the literature very little α-linked glucose is available for hydrolysis in the small intestine of ruminants fed on roughage diets. On high starch rations (ARMSTRONG and BEEVER, 1969; WALDO, 1973; GAILLARD and VAN 'T KLOOSTER, 1973) and dependent on physical form of the feedstuffs the quantity of α-glucose polymers reaching the small intestine of ruminants can make an appreciable contribution to the overall glucose supply (THOMPSON *et al.*, 1972; BEEVER *et al.*, 1972; THOMPSON and LAMMING, 1972). In cellulose and hemicellulose the sugar units are joined by β-linkages and are not degraded by mammalian enzymes. Breakdown of these sugars by bacteria present in the intestines, especially in the caecum and colon is likely to occur and will contribute to the amount of VFA absorbed daily form the gut.

In our department one of us (B.D.E.G.) initiated research on the relative digestion of carbohydrate fractions at different sites of the gastro-intestinal tract of cows (GAILLARD and VAN 'T KLOOSTER, 1973).

Samples from duodenal and ileal digesta collected in experiments designed to study

protein digestion or mineral absorption were analyzed for the contents of carbohydrate fractions. Recently a more detailed study of the carbohydrate composition of different fractions in intestinal digesta and faeces was made (GAILLARD and VAN 'T KLOOSTER, 1973).

In this communication results of some more experiments will be presented and discussed in relation to the ability of the intestine to digest and absorb sugars. Also the relative contribution of these sugars to the hexose and the gross energy supply of the animals will be considered.

EXPERIMENTAL

Animals, rations and sampling

The experimental animals with some details on the intestinal cannulas together with the composition of the rations fed in the different experiments are listed in table 1. Food was offered twice daily at intervals of about 12 h. Refusals were weighed and sampled. The preliminary feeding periods varied but were 10 days or more in most experiments. Samples were withdrawn from intestinal cannulas during 5 day periods in each experiment and bulked per cow for each site of the gut. From the T-piece can-

Table 1. Details of the different experiments. Single (T) or re-entrant (RE) cannulas were sited in the distal duodenum (DD) and/or distal ileum (DI). Ration composition is expressed as kg offered per day except for the grass rations.

Experiment		Intestinal cannulas		Rations expressed as kg offered/d		
Nr	Cow	DD	DI	hay	concentrates	other
1a	Hinke 14	T	RE	7	9	–
1b	Hinke 15	T	RE	9	1.5	–
2a	Anna	T	RE	7	3.5	–
2b	Hinke 15	T	RE	6	1	–
3a	Witschoft*	T	T	7	6	–
3b	Witschoft	T	T	3	3.7	3[a]
3c	Witschoft	T	T	–	–	grass[b]
4a	Cow 3	RE		8	8	–
4b	Cow 4	RE		7	8	–
5a	Cow 3	RE		–	–	grass (c)
5b	Cow 4	RE		–	–	grass[d]
6a	Frieda	T	RE	4	9	–
6b	Frieda	T	RE	11	3	–
6c	Hinke 15	T	RE	3	3	–
6d	Hinke 15	T	RE	10	–	–

* GAILLARD and VAN 'T KLOOSTER, 1973.

[a] paper pulp [b] 9.80 kg DM/d [c] 11.48 DM/d d 9.37 kg DM/d

nulas spot samples were taken every hour for 120 h (the day hours only) in the experiments with Witschoft and Zwartschoft and for 60 h (the day hours only) in the experiments with other cows. Digesta passing the re-entrant cannulas were sampled proportionally during 120 h in the experiments with cow 3 and cow 4 and during 60 h in the experiments with Hinke 14, Hinke 15, Anna and Frieda (day hours of a 5 day period).

Faeces were collected over 8 to 10 days. All samples of intestinal contents and faeces were freeze-dried. Samples of hay, concentrates and grass were dried at 60 °C before analysis.

Indicators

During 5 or more days preceding the collection periods and in the 5 days collection periods 100 g PEG and/or 5.25 g Cr_2O_3 were administered twice daily (VAN 'T KLOOSTER *et al.*, 1972). In the experiments with Frieda and Hinke (5a to 5d) and Anna and Hinke 15 (2a, 2b) PEG was pumped continuously into the rumen. When the two indicators were used in one experiment the results were calculated on both indicators and averaged (VAN 'T KLOOSTER and ROGERS, 1969).

Fractionation of carbohydrates (CH)

The CH were separated in fractions depending on their solubility in different solvents as outlined by GAILLARD and VAN 'T KLOOSTER (1973). As by the action of rumen micro-organisms part of the plant cell-wall may be only degraded to a lower molecular weight and therefore soluble at pH 7 without being completely fermented, a treatment with neutral detergent solution was avoided in later experiments (2a, 2b, 5a, 5b, 5c, 5d). After extraction of the ethanol-soluble carbohydrates, the α-glucose polymers and the water-soluble polysaccharides from feed, digesta samples and faeces (GAILLARD and VAN 'T KLOOSTER, 1973), 'hemicellulose' and 'cellulose' were determined in the residu. This residu was hydrolyzed with 2N H_2SO_4 in boiling water for 6 h and filtered. The filtrate was brought to volume and its sugar content was determined. The residue was dried with acetone and treated with 72% H_2SO_4 for 4 h at 20 °C ± 2. The mixture was diluted with 11 vol of water and further hydrolyzed in boiling water for 6 h. In the solution after filtration suger was determined.

The following fractions were obtained:

- ethanol-soluble carbohydrates (mono-, di- and oligosaccharides).
- α-glucose polymers (starch and microbial storage polysaccharides).
- other water-soluble polysaccharides (polysaccharides from glycoproteins, bacterial polysaccharides, fructosan, etc.).
- polysaccharides hydrolysable with 2N H_2SO_4 (hemicellulose of plant material and other easily hydrolyzable, water-insoluble polysaccharides). This fraction will be indicated as 'hemicellulose'.
- polysaccharides hydrolysable with 72% H_2SO_4 (cellulose and bacterial polysaccharides). This fraction will be indicated as 'cellulose'.

Analysis

In the extractions and hydrolysates total sugars were estimated after neutralisation by the method of HAGEDORN and JENSEN (1923) and the uronic acids by the carbazole method of DISCHE (1947). The sugar contents of polysaccharide-containing fractions were calculated as anhydro-sugars, i.e. α-glucose polymers and 'cellulose' as glucose × 0.9 and 'hemicellulose' as xylose × 0.88 to which was added the content of anhydro-uronic acid.

PEG and Cr_2O_3 were determined as described by VAN 'T KLOOSTER *et al.*, (1972). Gross energy (GE) was determined by combustion in a bomb calorimeter.

RESULTS

The quantities of CH consumed, their apparent digestibilities and the relative apparent digestibilities along the gut are given separately for each of the carbohydrate fractions in tables 2 to 6. The apparent digestibilities declined within the range: ethanol-soluble CH, α-glucose polymers, water-soluble CH, 'cellulose' and 'hemicellulose'. Only negligible amounts of ethanol-soluble CH were found in the faeces. The apparent digestibilities of ethanol-soluble CH (table 2) were not related to intake. This is not un-

Table 2. Intake of ethanol soluble carbohydrates and the sites of disappearance of apparently digested ethanol soluble carbohydrates in the gut of fistulated cows fed different rations.

Experiment		Intake	Apparent Digestibility	Disappearance in % of apparently digested		
Nr.	Cow	g/day	%	before duodenum	in small intestine	in caecum and colon
2a	Anna	434	94	93	3	4
2b	Hinke	181	92	83	13	5
3a	Witschoft*	442	93	83	11	6
3b	Witschoft	270	91	80	14	6
3c	Witschoft	860	96	93	7	1
4a	Cow 3	1202	98	92	–	–
4b	Cow 4	1114	98	91	–	–
5a	Cow 3	516	95	84	–	–
5b	Cow	421	96	85	–	–
6a	Frieda	808	97	–	–	6
6b	Frieda	797	97	–	–	2
6c	Hinke	350	98	–	–	4
6d	Hinke	697	97	–	–	1

* GAILLARD and VAN 'T KLOOSTER, 1973.

expected as the mono-, di- and oligosaccharides present in the food are most probably almost completely fermented in the forestomachs, while the sugar of this fraction present in digesta may stem from partly digested α-glucose polymers, mucus and from the microbes. Of the total apparent digestion of this fraction 8 to 22% (n = 5) took place in the intestines of which roughly 1/3 was acconted for in caecum and colon.

The apparent digestibilities of α-linked glucose polymers varied from 86 to 99% with the lower values on the grass rations, supplying only small amounts of α-glucose polymers (table 3). On these rations the relative digestion proximal to the duodenum was lower than on the hay and concentrate rations with considerably higher intakes of α-glucose polymer (80% versus 90%). Microbial storage polysaccharides contribute to the α-glucose polymers in the intestinal digesta. This contribution is likely to be of more importance at low intakes of α-glucose polymers. A considerable part of these polymers apparently escapes digestion in the small intestine. In the caecum and colon about 5% of the apparently digested α-glucose polymers disappeared, most probably by bacterial fermentation.

The water-soluble CH (table 4) in the faeces amounted to 11 to 34% of the quantities fed. These CH are most likely added to the digesta in the hind gut by mucus and bac-

Table 3. Intake of α-linked glucose polymers and the sites of disappearance of apparently digested α-linked glucose polymers in the gut of cows fed different rations.

Experiment		Intake g/day	Apparent Digestibility %	Disappearance in % of apparently digested		
Nr.	Cow			before duodenum	in small intestine	in caecum and colon
1a	Hinke 14	2469	98	86	11	3
1b	Hinke 15	384	94	93	6	1
2a	Anna	498	95	91	7	2
2b	Hinke 15	135	87	90	9	1
3a	Witschoft*	1687	96	73	20	7
3b	Witschoft	480	95	84	11	5
3c	Witschoft	199	87	74	6	20
4a	Cow 3	1596	98	87	–	–
4b	Cow 4	1584	98	92	–	–
5a	Cow 3	174	86	81	–	–
5b	Cow 4	145	88	80	–	–
6a	Frieda	2215	98	–	–	5
6b	Frieda	750	97	–	–	5
6c	Hinke 15	750	99	–	–	7
6d	Hinke 15	–	–	–	–	–

* GAILLARD and VAN 'T KLOOSTER, 1973.

Table 4. Intake of H_2O-soluble polysaccharides and the sites of disappearance of apparently digested H_2O soluble polysaccharides in the gut of fistulated cows fed different rations.

Experiment		Intake g/day	Apparent Digestibility %	Disappearance in % of apparently digested		
Nr.	Cow			before duodenum	in small intestine	in caecum and colon
2a	Anna	325	76	76	–4	28
2b	Hinke 15	160	66	63	0	36
3a	Witschoft*	730	89	76	18	6
3b	Witschoft	302	84	71	3	26
3c	Witschoft	300	83	63	12	25
6a	Frieda	456	87	–	–	17
6b	Frieda	436	77	–	–	9
6c	Hinke 15	210	84	–	–	19
6d	Hinke 15	440	84	–	–	4

* GAILLARD and VAN 'T KLOOSTER, 1973.

teria. The water-soluble CH of bacterial origin and of mucus may also have contributed considerably to the fraction in duodenal contents so that the true digestibilities of water-soluble CH proximal to the duodenum will be much higher than the apparent digestibilities. Digestion of this fraction in the small intestine was low but the contribution of caecum and colon to the apparent digestion was higher than found for any one of the other fractions.

In the experiments 6a to 6d the same roughage and concentrate mixture was fed in different proportions to the animals in a reversal experiment. The results suggest a stimulating effect of the concentrate proportion of the ration on the contribution of the caecum and colon to the digestion of water-soluble CH. However, the results of experiment 2a and 2b do not support this finding.

The apparent digestibilities of the 'hemicellulose' fraction varied in the different experiments from 53 to 81% (table 5). Distal to the duodenal cannulas hardly any digestion of this fraction was found, except in experiment 6a and 6c. Here the caecum and colon contributed substantially to the digestion.

Digestion of 'cellulose' was virtually completed before the digesta reached the small intestine (table 6). The contribution of the hind gut was too small in most of the experiments to be measured. In experiment 6b to 6d the contribution of caecum and colon was higher with the animals on concentrate rich rations. The apparent digestibilities were not affected clearly by the proportion of concentrate in the ration.

The anaerobic breakdown of CH in the forestomachs results in the production of VFA which form an important source of energy for the ruminant. As in the present experiments about 90% of the digested CH was fermented before the duodenum, is was

Table 5. Intake of 'hemicellulose' and the sites of disappearance of apparently digested 'hemicellulose' in the gut of fistulated cows fed different rations.

Experiment		Intake g/day	Apparent Digestibility %	Disappearance in % of apparently digested		
Nr.	Cow			before duodenum	in small intestine	in caecum and colon
1a	Hinke 14	2110	71	102	-3	1
1b	Hinke 15	1508	76	98	0	2
2a	Anna	1759	75	97	2	1
2b	Hinke 15	1287	77	101	3	-4
4a	Cow 3	1453	53	100	–	–
4b	Cow 4	1310	53	102	–	–
5a	Cow 3	1460	72	98	–	–
5b	Cow 4	1185	72	100	–	–
6a	Frieda	1459	71	–	–	13
6b	Frieda	2011	73	–	–	1
6c	Hinke 15	807	81	–	–	10
6d	Hinke 15	1670	75	–	–	0

Table 6. Intake of 'cellulose' and the sites of disappearance of apparently digested 'cellulose' in the gut of fistulated cows fed different rations.

Experiment		Intake of 'cellulose' g/day	Apparent digestibility %	Disappearance in % of apparently digested		
Nr.	Cow			before duodenum	in small intestine	in caecum and colon
1a	Hinke 14	1940	75	101	-1	0
1b	Hinke 15	1730	78	103	0	-3
2a	Anna	2122	81	98	1	1
2b	Hinke 15	1663	84	99	1	0
4a	Cow 3	1852	62	100	–	–
4b	Cow 4	1644	67	95	–	–
5a	Cow 3	2210	81	102	–	–
5b	Cow 4	1795	86	97	–	–
6a	Frieda	1072	73	–	–	7
6b	Frieda	1850	79	–	–	2
6c	Hinke 15	605	84	–	–	8
6d	Hinke 15	1680	81	–	–	-1

thought to be of interest to measure the relative apparent contribution of the forestomachs to the total GE supply. From table 7 it can be seen that only 40 tp 50% of the digested GE disappeared from the forestomachs. The small intestine contributed for 50 to 60%, while the contribution of the hind gut varied between 1 and 12%.

If the energy content of (poly)saccharides is assumed to be 4.2 kcal/g the quantity of GE represented by the quantity (g/day) of CH apparently fermented in the forestomachs can be calculated. It was calculated that the measured quantity of GE, disappearing proximal to the duodenum, was only about 65% of the GE present in the CH fermented in the forestomachs. This finding can partly be explained by the relatively high lipid content of duodenal contents compared with hay and concentrates. This is due partly to microbial lipid synthesis in the forestomachs. In 6 experiments (1a, 1b, 4a, 4b, 5a and 5b) the quantities of etherextractable matter present in duodenal digesta surpassed the quantities analysed in the rations fed by 570 ± 54 g per day. As the duodenal cannulas were fitted distal to the inflow of the bile duct, part of the lipid fraction in duodenal contents stems form bile substances as lecithine, bile salts and porphyrines. Combustion heat of bile from slaughter cows was estimated at 6.8 ± .13 kcal/g dry matter (n = 6). The energy content of duodenal organic matter was indeed higher than the energy content of organic matter of the rations fed, i.e. 5.7 and 4.8 kcal/g, respectively.

Table 7. Gross energy (GE) of rations fed to fistulated cows and the sites of disappearance of GE from the gut.

Nr.	Cow	GE intake kcal/d	Total Disappearance %	Disappearance in % of total disappearance		
				before duodenum	in small intestine	in caecum and colon
1a	Hinke 14	58042	68	39	60	1
1b	Hinke 15	36642	65	44	55	1
2a	Anna	38690	64	40	53	7
2b	Hinke 15	25577	62	48	49	3
4a	Cow 3	56833	62	45	–	–
4b	Cow 4	52867	64	49	–	–
5a	Cow 3	47112	64	42	–	–
5b	Cow 4	38085	69	43	–	–
6a	Frieda	53396	78	–	–	8
6b	Frieda	49450	72	–	–	5
6c	Hinke 15	38080	69	–	–	1
6d	Hinke 15	22474	79	–	–	12

Discussion

The CH reaching the small intestine can be divided in two groups based on their digestibility i.e. one group containing water-soluble CH, 'hemicellulose' and 'cellulose' and a second group containing ethanol-soluble CH and α-linked glucose polymers. In the former group the constituent sugars are linked by β-linkages, which can not be hydrolyzed by mammalian enzymes. The breakdown in the intestines depends on the presence of bacterial enzymes. The latter group contains sugars that, when bound, have linkages which can be hydrolyzed by pancreatic and intestinal enzymes. McAllan and Smith (1974) showed that more than half of α-glucose polymers entering the duodenum of a ruminating calf are derived from the rumen microbes when feeding rations consisting of equal weights of hay and cereals. These authors reported contents of α-glucose polymers in rumen microbes from 25 to 160 g per kg dry bacterial cells, depending on the proportion of concentrates in the diets. Even higher α-glucose polymer contents of rumen bacteria separated from the rumens of cows receiving diets high in digestible carbohydrate are reported by Jouany and Thivend (1973) (over 200 g/kg dru matter). These results show that microbial synthesis of storage polysaccharides may contribute considerably to the digestable carbohydrates entering the duodenum, especially when high concentrate rations are fed. With the grass rations of experiments 3c, 5a and 5b 400 to 600 g of fructosan and only 150 to 200 g of starch were fed. It is most likely that with these low starch intakes almost all α-glucose polymers present in duodenal contents were of microbial origin.

With hay and concentrate rations the quantity of α-glucose polymers in duodenal contents amounted to 9 to 27% of the starch present in the rations. These results are in general agreement with other published results recently reviewed by Waldo (1973). It has been shown that the form of the starch source (Mayes and Ørskov, 1974) and particle size of the roughage component of the diet may also have a significant effect upon the amounts of starch that escape fermentation in the rumen of sheep (Thompson and Lamming, 1972).

Pancreatic amylase is largely responsible for the hydrolysis of starch in the intestine. This enzyme splits α-1.4 linkages in glucose polymers, producing maltose and isomaltose. As in monogastric animals only small amounts of glucose are produced from starch by pancreatic juice. Siddons (1968) has shown that pancreatic juices from mature cattle have high amylase and weak maltase activities. In calves 14–16 weeks old amylase activities in pancreatic juices were lower but still appreciable, while in other experiments the amylase activities in pancreatic juices of 1 week old calves proved to be very low (Turnouth and Buttle, 1973). Clary *et al.* (1967) found increased secretion of pancreatic amylase in sheep receiving high starch rations for extended periods. Such adaptations may explain the relative low amylase activities reported by Keller *et al.* (1958) in bovine pancreatic juice. However Mayes and Ørskov (1974) infused starch into the abomasum of lambs and mature sheep over periods of 4 weeks and found a low net disappearance of total α-linked glucose polymers (58%) with no ten-

dencies for the percentage disappearance values to change with time. This suggests that adaptation in starch utilization as a response to starch in the small intestine does not occur. In the same experiments it was noticed that starch was very effectively digested to oligosaccharides, whereas the percentage disappearance of oligosaccharides was low. This may suggest that α-amylase was not limiting the digestion of starch but probably maltase. That the ability of the small intestine of ruminants to digest starch is limited has been found in experiments with steers (KARR *et al.*, 1966; LITTLE *et al.*, 1968). The results of the present experiments with cows confirm those findings as the apparent digestibility of α-glucose polymers in the small intestine was found to be less than 50% (of the quantities of α-glucose polymers present in duodenal digesta) when the starch intake was low (2a, 2b, 1b). Higher percentages but still low (60–65%) were found when starch intakes were 1.5 to 2.5 kg per day. LITTLE *et al.* (1968) and MCALLAN and SMITH (1974) found about the same percentages (42–65%) for the digestibility of α-linked glucose polymers in the small intestine of cattle.

The complete breakdown of starch to glucose and the hydrolysis of disaccharides, require the intestinal disaccharidases capable of splitting both α-1.4 and α-1.6 linkages, as well as the β-galactosidic linkages in lactose. Different groups of workers (HEMBRY *et al.*, 1967; COOMBE and SIDDONS, 1973) have demonstrated the presence in mucosal homogenates of sheep and cattle of maltase, isomaltase, trehalase, lactase and cellobiase activities. The maltase and isomaltase activities found were low compared with the activities found in the small-intestinal mucosa of some other mammals. This may explain the low ability of the small intestine to use starch. COOMBE and SIDDONS (1973) produced evidence that lactase and cellobiase activities were brought about by the same enzyme. Both activities declined with age of the calves. It is not known if cellobiose is present in duodenal contents, but if so then most probably in very small amounts only.

Studies to the ability of the ruminant small intestine to absorb monosaccharides are scanty (HEMBRY *et al.*, 1967). WHITE *et al.* (1971) studied the absorption of glucose in different parts of the small intestine in acute experiments with sheep and concluded that glucose was absorbed by passive and active transport, but it was not possible to assess from their work the contribution each mechanism makes to glucose absorption. Like in different monogastric animals, including man, the rate of absorption of glucose was higher in the proximal jejunal region than in the ileal region. The between – species comparisons of the absorptive capacity of the small intestine per kg $W^{0.75}$ based on first order kinetics showed 5.3 times greater absorption in the rat than in grazing sheep and 2.8 times greater than in sheep given wheat and lucerne chaff. The faster glucose absorption in sheep fed concentrates compared with grazing sheep and the increased ability of the small intestine in lambs on high lactose intakes to absorb glucose, was supposed to be due to adaptation at the cellular level.

Experiments with monogastric animals have shown that the rate of absorption of glucose and galactose is about 5 or more times greater than the absorption of other hexoses, like mannose, and of pentoses. This may also apply in the ruminant. Sugars

other than glucose and galactose, if present in intestinal contents are probably not absorbed to any degree but are possibly metabolized by bacteria. With the low disaccharidase activities together with a relatively slow absorption of glucose and possibly other sugars (galactose shares a common carrier with glucose) it is quite explainable that the efficiency of absorption of ethanol-soluble sugars and α-glucose polymers will be low. In the present experiments less than 100 g of mono-, di-, oligosaccharides and α-glucose polymers were absorbed from the small intestine of cows when the rations were composed of grass or of hay and relatively low amounts of concentrates. With the high concentrate rations the intake of these sugars amounted to 2 to 3 kg per day. On these rations, fed to cows producing 20 to 25 kg milk per day, the quantities of the same sugars disappearing from the small intestine amounted to 200 to 400 g per day. As the glucose requirement of these latter cows can be estimated roughly at 1.9 to 2.3 kg per day (see introduction) the glucose absorption from the small intestine at high starch intakes is likely to contribute about 10 to 20% of the total glucose requirement. Even when allowance is made for the rough nature of these calculations it is apparent that glucose synthesis from nonhexose precursors as propionic acid is a much more important source of glucose than hexose absorbed from the intestine.

A detailed study of the water-soluble CH in diets, in intestinal contents and in faeces of cows was made by GAILLARD and VAN 'T KLOOSTER (1973). A comparison between the relative proportions of sugars in the water-soluble CH fraction of the diets and duodenal digesta showed increases of sialic acids, galactose, fucose and rhamnose but decreases for glucose and or xylose. These changes could be explained by additions of polysaccharides form mucus and bacteria. Glycoproteins of mucus are mainly composed of sialic acids, aminosugars, galactose, mannose and fucose while bacterial polysaccharides contain xylose, arabinose, rhamnose, mannose, galactose, glucose and aminosugars (HOOGENRAAD and HIRD, 1970). MCALLAN and SMITH (1974) estimated the contribution of microbial carbohydrates in duodenal contents from the ratio of individual sugars to nucleic acids in samples of mixed rumen bacteria and in samples of duodenal contents. They concluded that nearly all the rhamnose, mannose and ribose and about half the galactose and glucose came from bacterial synthesis, whereas nearly all the arabinose, xylose and cellulose-glucose was contributed by the diet.

Changes in the amount and in the proportions of individual sugars in the water-soluble CH fraction occurred during passage through the small intestine, caecum and colon (GAILLARD and VAN 'T KLOOSTER, 1973) possibly by addition of mucus to the digesta and microbial metabolism. A net fermentation of this carbohydrate fraction was noticed in caecum and colon (table 4).

In the present experiments the apparent digestion of 'hemicellulose' as well as of 'cellulose' was almost completed before the digesta reached the duodenum (table 5 and 6). The same conclusion was drawn form earlier experiments with cows fed different rations (GAILLARD and VAN 'T KLOOSTER, 1969). In experiments with sheep a significant post-ruminal digestion of hemicellulose has been reported especially on rations in which the roughage was fed ground and pelleted (ARMSTRONG *et al.*, 1969: BEEVER

et al., 1971; THOMSON *et al.*, 1972). Grinding and pelleting of roughage not only reduced the relative contribution of the forestomachs but also depressed the overall digestibility. From the sheep experiments mean apparent digestibility percentages of 73 en 70 were calculated for hemicelluloses in roughages in the long form and ground and pelleted roughages, respectively. The relative contribution of the forestomachs in hemicellulose digestion in those experiments amounted to 81 and 67% respectively, while the caecum and colon contributed 28 and 17%. The difference in the contribution of the caecum and colon of cows as compared with sheep may point to species differences.

The finding that the 'cellulose' digestion was almost restricted to the reticulorumen of the cows in the present experiments, is consistent with the results of GAILLARD and VAN 'T KLOOSTER, 1969; MITCHELL *et al.* (1967) and WATSON *et al.* (1972). Extensive post-ruminal digestion of cellulose in both sheep and calves have been reported by WARNER *et al.* (1972). They infused from 200 to 600 g/day purified wood cellulose into the abomasum or caecum of fistulated animals and found that about 1/3 of the cellulose disappeared from the intestine. However the cellulose escaping digestion in the rumen might be expected to be more resistent to digestion than the purified cellulose infused in those experiments. Disappearance of cellulose posterior to the reticulorumen has been shown to occur in sheep (ULYATT, DELLOW, REID and BAUCHOP, 1975; ARMSTRONG and BEEVER, 1969). When sheep are fed hay or grass either fresh or dried, the post-ruminal fermentation of cellulose is relatively small and generally does not exceed 10% of the total amount digested. However, digestion of cellulose in the reticulorumen was reduced by grinding and pelleting of the roughage and this was partly compensated for by increased digestion in the caecum and colon (ARMSTRONG and BEEVER, 1969; BEEVER *et al.*, 1971; 1972; THOMSON *et al.*, 1972). Other factors influencing cellulose digestion are the proportion of concentrates and high fat contents of the rations fed. In experiments 6a to 6d the contribution of the caecum and colon was found to be greater with the high concentrate rations as suggested in earlier experiments (GAILLARD and VAN 'T KLOOSTER, 1969).As the error involved in these estimates is considerable, the differences found were not significant. ULYATT *et al.* (1975) showed that as apparent digestibility of cellulose decreased the proportion of cellulose digested in the large intestine increased.

The proportion of digestible energy that was digested in the stomachs ranged from 38 to 49% in the present experiments. Higher (BEEVER *et al.*, 1972; WATSON *et al.*, 1972) as well as lower percentages (THOMPSON *et al.*, 1972; TAMMINGA, 1975) have been reported. Part of these differences is caused by sampling of the digesta at different sites of the duodenum. Samples withdrawn from the distal duodenum as in the present experiments contain pancreatic juice and bile. This reflected the considerable increase in energy concentration of the organic matter between intake and duodenal digesta. Other factors that seem to influence the proportion of digested energy that disappears proximal to the duodenum are: physical form of the roughage fed (THOMSON *et* al., 1972), the proportion of roughage in mixed rations (KAUFMANN *et al.*, 1973) and the

amount of dry matter intake above maintenance level (TAMMINGA, 1975). Further, variations in the storage polysaccharide content of microbial cells, in the lipid content of microbial preparations (ØRSKOV *et al.*, 1971) and in the quantity of protein synthesized in the forestomachs can effect the relative disappearance of GE from the gut proximal to the duodenum.

In ruminants digestion and absorption of protein (VAN 'T KLOOSTER and BOEKHOLT, 1972) and of lipid substances (LEAT and HARRISON, 1975) occurs mainly in the small intestine. This together with digestion and absorption of α-glucose polymers, ethanol soluble CH and other substances such as nucleic acids and bile acids probably equals the disappearance of apparently digested GE from the small intestine.

In the present experiments the digestion in the caecum and colon contributed only slightly to the energy requirement of the cows. This is consistent with the relatively low contribution of this site to the digestion of CH. However, the disappearance of GE from the caecum and colon was about 2 times as high as the GE calculated from the quantity of CH digested (g × 4.2 kcal.). It is not clear what substances were being fermented besides the CH but small quantities of protein and lipid substances disappeared in the caecum and colon, probably due to digestion by bacteria.

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CHANGES IN THE COMPONENT MONOSACCHARIDE RATIOS OF GALACTOMANNANS FROM LEGUMINOUS SEEDS DURING MATURATION[1]

P. KOOIMAN

Laboratory for General and Technical Biology[2],
Delft University of Technology, The Netherlands

From ripe and from unripe seeds of six leguminous species galactomannans were isolated. The mannose-to-galactose ratios of the purified glactomannans were determined using gas chromatography of the acetylated alditols, obtained by hydrolysis of the polysaccharides, reduction of the monosaccharides and acetylation of the latter. The mannose-to-galactose ratios were markedly higher in the galactomannans from the ripe seeds of all the species. It was concluded that galactomannans in ripe leguminous seeds having a mannose-to-galactose ratio larger than one have been stripped of part of their galactose side chains during the maturation of the seeds.

INTRODUCTION

In a previous publication (KOOIMAN, 1971) the question was raised if the mannose-to-galactose ratios in galactomannans of leguminous seeds are constant during maturation, or if there is a gradual diminishing of the galactose content. In some *Palmae* a drastic lowering of the galactose content has been observed leading to an end product commonly designated as a mannan.

No change in the mannose-to-galactose ratio was found during ripening of seeds of *Trigonella foenum-graecum* (REID and MEIER, 1970), whereas a small increase in this ratio was observed when the galactomannan from ripe seeds of *Gleditschia ferox* was compared with the same from unripe seeds of this species (COURTOIS and LE DIZET, 1963). It was deemed useful to investigate this ratio during ripening of some other leguminous species in order to get more certainty on this point. The present communication presents the results of this investigation.

[1] The skilful technical assistance of Miss D. C. REUVERS is gratefully acknowledged.

[2] Julianalaan 67, Delft.

MATERIALS AND METHODS

Sources of galactomannans

Seeds were harvested from the following species which were grown in the 'Cultuurtuin voor Technische Gewassen' of the Technological University at Delft: *Crotalaria lanceolata* E. Mey., *Cytisus* × *praecox* Bean, *Cytisus scoparius* Link, *Genista tinctoria* L., *Spartium junceum* L., and *Ulex europaeus* L.

As a criterion for the state of maturity the following parameters were used: percentage of dry material of the empty pods, percentage of dry material of the seeds, and dry weight per seed. Drying was performed at 60 °C. The respective data are recorded in table 1.

Extraction and purification of galactomannan

The seeds were treated with boiling ethanol directly after harvesting. After drying the seeds were ground and extracted with ethanol-benzene (1:1). The ground seeds were extracted several times with boiling water and the combined filtrates treated with two volumes of ethanol. The crude galactomannan which precipitated was separated from the supernatant, washed with 70% ethanol, and dried by solvent exchange and then in vacuo. The product (100 mg) was dissolved in water (20 ml) and the solution was centrifuged to remove any insoluble material. Fehling's solution (.25 ml diluted to 2.5 ml) was added and the precipitated copper complex was separated and washed

Table 1. Mannose-to-galactose ratios in leguminous galactomannans.

plant species	mature (m) immature (i)	dry weight of empty pods (%)	dry weight of seeds (%)	dry weight per seed (mg)	yield of crude galactomannan (%)	yield of purified galactomannan (%)	mannose-to-galactose ratio
Crotalaria lanceolata	i	37	33		13.9		2.10
Crotalaria lanceolata	m	*c.*90	*c.*90		17.3		2.42
Cytisus praecox	i	74	*c.*45	*c.*5	2.2	.5	1.26
Cytisus praecox	m	*c.*90	*c.*90	*c.*8		.8	1.82
Cytisus scoparius	i	38	46	7.4	11.3	6.2	1.68
Cytisus scoparius	m	91	94	7.4	3.2	1.5	1.92
Genista tinctoria	i	45	49	5.2	4.2	1.7	1.63
Genista tinctoria	m	91	96	4.3	4.3	1.0	1.85
Spartium junceum	i	34	40	11.3	3.3	1.1	1.38
Spartium junceum	m	*c.*90	95	15.2	1.9	.3	1.84
Ulex europaeus	i	46	45	6.8	1.7	.3	1.34
Ulex europaeus	m	89	96	6.7	7.8	1.9	1.41

with water. The precipitate was suspended in water (10 ml) and .2M hydrochloric acid (1 ml) was added to destroy the copper complex. The regenerated galactomannan was precipitated with ethanol, washed and dried.

Hydrolysis of the purified galactomannan

Galactomannan (10 mg) was heated for 7 hours in a sealed tube with nitric acid (1 ml) in a boiling water bath. The acid was removed using anion exchange resin Amberlite IR45.

Reduction of the monosaccharides and acetylation of the alditols

The monosaccharide mixture was reduced to the corresponding alditol mixture by addition of an equal weight of potassiumborohydride to the aqueous solution and leaving the solution for 16 hours to react. Excess of borohydride was then destroyed by the addition of dilute acetic acid and the solution was deionized by a mixture of Amberlite IR120 and 45 ion exchange resins (1:1). The alditol solution was concentrated to dryness at reduced pressure and methanol was added. The methanol was evaporated and the process of methanol addition and evaporation was repeated three times in order to remove any residual borate. The alditol mixture was acetylated by adding .2 ml pyridine and .2 ml acetic anhydride and heating the solution in a sealed tube at 110 C for 90 minutes. The solvents were removed in a desiccator over silica gel.

Determination of the alditol acetates

The acetylated alditols were dissolved in chloroform (2 ml) and a small portion (3–4 μl) was injected into the gaschromatograph. Gaschromatography was performed in a Hewlett-Packard apparatus type 5750. A glass column (diameter .3 cm, length 250 cm) was used filled with 3 %OV-275 on 100/120 Chromosorb W. The chromatograph was operated at 225 C and gas (nitrogen) velocity of 20 ml per minute.

Under these conditions the retention times of galactitol acetate and mannitol acetate were 467 and 422 seconds, respectively. The ratio of mannose to galactose was determined by estimation of the ratio of the peak areas of mannitol acetate and galactitol acetate. Recorded values are averages of duplicate estimations (table 1). Previous to the determinations of the sugar ratios in the galactomannan hydrolysates model experiments were performed including subjecting free sugars to the hydrolytic conditions used for the polysaccharides, reductions and acetylations of known mixtures of sugars and preparation of pure alditol acetates for reference[3].

[3] A sample of crystalline D-xylitol acetate was generously supplied by Professor E. SJÖSTRÖM (Otaniemi, Finland) and used for inoculation of a xylitol acetate syrup.

RESULTS AND DISCUSSION

The quantities of seeds harvested for the present study ranged from 1 to 15 grams (dry weight) per species. Because for these quantities hundreds of pods were required it was not possible to register the numbers of days after anthesis as a criterion of the state of maturity of the seeds. In order to have an indication of the latter parameter the percentages of dry material of the empty pods and of the seeds as well as the dry weight per seed have been determined. It should be emphasized that as a consequence the data for the immature seeds represent average values of more or less widely diverging states of immaturity. Furthermore it is notable that in one species (*Cytisus praecox*) the difference between the dry weights of immature and of mature pods is comparatively small, whereas the difference between the dry weights of immature and of mature seeds is large. A similar disparity between the dry weight percentages of the seeds and the dry weights per seed was observed in some other species. The immature seeds of *Cytisus praecox* and of *Spartium junceum* have evidently been taken in an early state so that they had not yet reached the final quantity of biomass. In *Cytisus scoparius* and in *Ulex europaeus* there is virtually no difference in dry weight between the immature and he mature seeds, although both the immature pods and seeds contained still more than 50% water in their tissues. The seeds of *Genista tinctoria* lost some of the dry material during the process of ripening.

The yields of crude galactomannans were low as compared to those of the well-known sources of galactomannans *Ceratonia siliqua* and *Cyamopsis tetragonolobus* where the yields are up to 50%. During purification *via* the copper complex much of the starting material was lost; it was considered to consist of polymers other than galactomannan. REID and MEIER (1970) pointed out that leguminous galactomannans with high as well as with low mannose-to-galactose ratios form insoluble copper complexes and they demonstrated for *Trigonella foenum-graecum* that the sugar composition of the galactomannan did not change on purification *via* the copper complex. It remains, however, possible that other galactose- or mannose-containing hemicelluloses, if present in unripe seeds, influence the results.

The mannose-to-galactose ratios of the galactomannans from immature and from mature seeds show marked differences, the galactomannans from the mature seeds having lower galactose contents than those from the immature seeds. The galactomannans of the species investigated in the present study behave therefore differently from the galactomannan of *Trigonella* (REID and MEIER, 1970), where the galactomannan maintained a constant composition during ripening. On the other side, the behaviour of the galactomannans is in agreement with that observed by COURTOIS and LE DIZET (1963) who found a difference (3.73 in cold water extracted galactomannans from unripe *vs* 3.90 in those from ripe seeds) in the mannose-to-galactose ratio of galactomannans from *Gleditschia* seeds. The situation parallels, though much less extremely, the loss of galactose from galactomannan during maturation of palm seeds, where the ultimate product is usually called a mannan and contains but a few percent of galactose.

It is evident that species where the ripe seeds have a galactomannan with a mannose-to-galactose ratio of 1:1 (like *Trigonella*) lack the mechanism resulting in a loss of galactose during ripening: with the leguminous type of structure a galactomannan could not have a smaller ratio. Species in which this situation prevails belong particularly to the tribes *Trifolieae* and *Loteae* of the subfamily *Faboideae* (*Leguminosae*) (HEGNAUER, 1957; REID and MEIER, 1970; KOOIMAN, 1972). In species where the ultimate ratio is larger than 1:1 probably an α-galactosidase operates during maturation and the galactomannan of the unripe seed has a ratio which is smaller, viz. nearer to 1:1. It is reasonable to assume that the liberated galactose is consumed at the benefit of the embryo. An extension of this thought leads to the assumption that the embryos of the leguminous species lacking endosperm in their ripe seeds have consumed all of the galactomannan before maturation. Be this an assumption, it has been demonstrated that the endospermless leguminous species do have important quantities of endosperm in the earlier stages of seed development and that this endosperm is resorbed during growth of the embryo (for references see HEGNAUER, 1957). It has also been demonstrated that in *Trigonella* galactomannan is deposited in the endosperm cell-walls in an early stage of maturation (at least 5 weeks after anthesis, the seeds being ripe 15 weeks after anthesis) (REID and MEIER, 1970, 1973).

On the basis of the results obtained in the present study it is not possible to make a statement on the mannose-to-galactose ratio of the galactomannan as it is synthesized in the unripe endosperm[4]. In some of the investigated species (*Cytisus praecox*, *Spartium junceum*) an initial ratio of 1:1 seems probable. It is an attractive hypothesis that the leguminous-type galactomannans when synthesized have a monosaccharide ratio of 1:1 and that after galactomannan has been deposited in the endosperm cell-walls the process of galactose removal becomes operative in the species where the ultimate ratio is larger than 1:1. More certainty on this problem can be obtained by estimating the mannose-to-galactose ratios of the galactomannans from a series of seed samples of well-defined states of immaturity for a number of species. Then also some insight may be obtained concerning the activity of α-galactosidase during the process of ripening.

[4] It is remarkable that, in contradistinction to the amyloids, which occur either in endosperm or in cotyledons (KOOIMAN, 1960), the galactomannans have so far been found exclusively in endosperm (KOOIMAN and KREGER, 1960; DEA and MORRISON, 1975). In the *Convolvulaceae* the galactomannan of *Convolvulus tricolor* seeds also occurs exclusively in the endosperm (personal observation).

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RELATION BETWEEN DIGESTIBILITY AND COMPOSITION OF FEED*

D. J. MINSON

Division of tropical Agronomy, C.S.I.R.O., Cunningham Laboratory, St. Lucia, Queensland, Australia, 4067

SUMMARY

Literature is reviewed on the potential digestibility of different fractions of the plant and a theoretical model of potential digestibility is proposed in which the plant is divided into four main fractions.

1. The group of readily soluble and readily hydrolysable carbohydrates, proteins, minerals and some cuticle which may, for practical purposes be regarded as being potentially fully digestible.

2. The higher molecular carbohydrates consisting mainly of cellulose and hemicellulose that are free of protection and which are potentially fully digestible.

3. The higher molecular carbohydrates which are fully protected by encrustations of lignin, cuticle or other resistant fractions and hence are completely indigestible.

4. Lignin, silica and cuticle which are indigestible.

Separation of some of these fractions may be achieved chemically but the separation of fractions 2 and 3 can only be achieved by bioassay methods involving prolonged digestion with rumen microorganisms.

All of the potentially digestible fraction of the food is not usually digested by animals. The factors controlling this loss of potentially digestible material include particle size of the feed, level of feeding, animal species, intestinal parasites and nutrients essential for the rumen microflora, particularly sulphur and nitrogen. The *in vivo* digestibility of feeds was further reduced by unavoidable endogenous excretions.

It was concluded that the *in vivo* digestibility of feeds was controlled by so many factors that it is more desirable at this time to have a simple model of potential digestibility and to describe the factors causing deviations from this model than to attempt to develop one comprehensive regression for predicting *in vivo* digestibility under all circumstances.

INTRODUCTION

The apparent digestibility of a ration is the percentage difference between the quantity of food consumed and faeces produced. It is determined by feeding 'the experimental ration in exact quantities for long periods, in order to ensure that a "steady state" of faecel excretion is reached, and then to colect the faeces excreted during a measured interval of time' (BLAXTER, *et al.*, 1956). The apparent digestibility of a feed

* This is a report.

by animals (A) is the potential digestibility of the feed (P) minus the quantity of the potentially digestible fraction of the feed which escaped digestion in the animal (E) and the unavoidable metabolic excretions (M).

$$A = P - (E + M)$$

The true digestibility of a feed by animals (T) is the apparent digestibility of the feed plus the unavoidable metabolic excretions (M). True digestibility therefore makes no allowance for the inability of the animal to completely digest the potentially digestible fraction. Thus: -

$$T = A + M$$

$$\text{and } P = T + E$$

A knowledge of these factors and their interaction should eventually lead to the development of more reliable methods of predicting digestibility and to a greater understanding of the underlying limitations to digestibility data.

In this paper the feed composition and animal factors controlling digestibility are reviewed together with evidence on the interactions between these two factors.

Potential digestibility

The concept of potential digestibility was developed by Wilkins (1966, 1969) who defined it 'as the maximum digestibility attainable when conditions and the duration of fermentation are not limiting factors'. The potential digestibility of some plant fractions may be determined with animals since the fractions are completely or almost completely digested. *In vivo* measurements may also be used to identify the fractions which are completely indigestible since any fraction found to have a very low digesibility *in vivo* is unlikely to become digestible simply by prolonging digestion time. However, the potential digestibility of the cellulose and hemicellulose of the cell wall can only be measured with special prolonged digestion techniques (Wilkins, 1966, 1969).

Soluble carbohydrates

The potential digestibility of the soluble carbohydrates in forages may be determined in *in vivo* digestibility studies since faeces contain little or no soluble carbohydrates. Gaillard (1962) found that the monosaccharides, disaccharides, fructosan and starch in a wide range of feeds were completely digested by ruminants (Table 1). Complete or almost complete digestion has also been found when the forages were analysed for cytoplasmic and soluble carbohydrate fractions (Jarrige, 1960; Jarrige and Minson, 1964; Waite *et al.*, 1964) (Table 1).

Table 1. Potential digestibilities of feed fractions

Fraction	Diet	Digestibility (in %)		Reference
		Mean	Range	
Monosaccharides	Grasses	100	–	Gaillard 1962
Disaccharides	Grasses	100	–	Gaillard 1962
Fructosan	Grasses	100	–	Gaillard 1962
Cytoplasmic carbohydrates	Legumes	99.1	98.2–100	Jarrige 1960
	Grasses	99.1	98.8–99.4	Jarrige 1960
Soluble carbohydrates	Grasses	99.2	95.2–99.9	Jarrige and Minson, 1964
	Grasses	100	–	Waite et al., 1964
Crude protein	Mixed Pasture	94.8	–	Dijkstra, 1954
	Mixed Pasture	93	–	Holter and Reid, 1959
	Mixed Pasture	88	78–95	Sullivan, 1964
	Mixed Pasture	90	–	Milford and Minson, 1965
	Mixed Pasture	93	–	Pigden, 1969
	Legumes	92	–	Combellas et al., 1971
	Grasses	97	–	Combellas et al., 1971
Neutral detergent solubles	Mixed Pasture	98	–	Van Soest and Moore, 1965
	Panicum	91	–	Minson, 1971
	Mixed Pasture	83		Combellas et al., 1971
	Mixed Pasture	101	–	Deinum, 1973
Cellulose	Solka Floc*	100	–	Donefer, Crampton & Lloyd 1960
	Lolium perenne	77.2	70.0–87.9	Wilkins, 1969
	Dactylis glomerata	72.6	62.8–82.3	Wilkins 1969
	Chloris gayana	59,8	49.2–74.1	Wilkins 1969
Hemicellulose Lignin	Straw	55	50–60	McAnally, 1962
	Mixed Pasture	1.5	0.7–2.2	Crampton and Maynard, 1938
	Medicago sativa	8.8	0.4–17.1	Gaillard 1962
	Trifolium pratense	1.0	–	Gaillard 1962
	Grasses	1.6	-7.4–10.6	Gaillard 1962
	Lupinus	18.1	–	Gaillard 1962
	Lolium perenne	0.1	-21.0–17.4	Jarrige and Minson, 1964
	Dactylis glomerata	0.2	-29.0–21.9	Jarrige and Minson, 1964
	Chloris gayana	2.6	–	McLeod and Minson, 1974
	Setaria splendida	-1.4	–	McLeod and Minson, 1974
	Lolium perenne	-24.2	–	McLeod and Minson, 1974
Silica	Mixed Pasture	3.4	–	Jones and Handreck 1965
	Medicago sativa	1.1	–	Jones and Handreck 1965
	Avena sativa	0	–	Jones and Handreck 1965
Cuticle	–	–	–	No data published

* Unlignified purified cellulose.

Crude Protein

The simple *in vivo* method cannot be used to estimate the potential digestibility of crude protein because faeces contain metabolic products of similar composition to the feed fraction being determined. This problem may be overcome (BLAXTER and MITCHELL, 1948) and the rue digestibility of feed protein calculated by a regression technique relating the apparent *in vivo* digestible crude protein % of the feed (Y) to the crude protein % of the feed (X). The true digestibility of the crude protein is the regression coefficient in the equation $Y = a + bx$ where a is the quantity of faecal protein of non feed origin.

Using this approach the true digestibility of the crude protein in herbage has varied from 88 to 95%. So little feed protein appears in the faeces (MASON, 1971) that the potential digestibility of feed protein will be approximately equal to the estimated true digestibility (Table 1). The difference in true or potential digestibility of protein may reflect real differences in digestibility between the feeds used in the different studies but is more probably influenced by the method of preparing the faeces for analysis because nitrogen is lost when faeces are dried (RAYMOND and HARRIS, 1954).

Neutral detergent solubles

The fraction soluble in neutral detergent contains the sugars, water soluble carbohydrates, starch, non-protein nitrogen, lipids and a proportion of the protein and minerals of the diet (VAN SOEST, 1967). The potential digestibility of this fraction has been determined by the regression method (described above) and found to vary from 83 to 98% (Table 1).

Cellulose and hemicellulose

The potential digestibility of cellulose in forage has been determined by prolonged incubation of feed samples *in vitro* (Table 1), or prolonged digestion in nylon bags suspended in the rumen (WILKINS, 1969). The cellulose digestibility of forage samples suspended in the rumen for 6 days did not differ significantly from cellulose digestibility measured after 6 days incubation *in vitro* when all remaining cellulose was found to be lignified or cutinized (WILKINS, 1969). MCANALLY (1942) found that the potential digestibility of hemicellulose in wheat straw varied between 50 and 60%.

Pure cellulose, free of any protective lignin, is completely digested *in vitro* (DONEFER *et al.*, 1960).

Lignin

The cell wall consists of a net-like structural framework of cellulose interspersed with globular particles of hemicellulose and encrusted to varying extents by lignin (FENGAL, 1971). The lignin polymers are chemically stable structures which can only be attacked by aerobic bacteria and fungi. It is therefore unlikely that lignin can be extensively broken down under the anaerobic conditions of the digestive tract (KEFFORD, 1958). In most *in vivo* digestion studies lignin has a very low digestibility (Table

1) although both positive and negative digestibilities have been reported. The existence of negative digestibilities is due to lignin artefacts produced during drying of the feed and faeces (NORMAN and JENKINS, 1934) while positive digestibilities may be due to the formation of lignin-carbohydrates complexes in the rumen (GAILLARD and RICHARDS, 1975).

Other indigestible fractions

Silica and cuticle are indigestible plant fractions that may affect digestibility. JONES and HANDRICK (1965) found that between 96.6 and 100% of silica in the feed was recovered in the faeces (Table 1). However, other studies have shown incomplete recovery but these have been attributed to unsatisfactory methods of analysis (JONES and HANDRICK, 1967). Plant cuticle is present in the faeces of sheep in a form that often allows the plant species being eaten to be identified (HERCUS, 1960). No quantitative data have been published on the potential digestibility of cuticle but the excretion of readily identifiable fragments in the faeces indicates that some cuticle is very resistant to digestion. However, SLATER and JONES (1971) were unable to detect microscopically any cuticle in the faeces of sheep fed *Trifolium repens*.

SUMMATION MODEL OF POTENTIAL DIGESTIBILITY

The potential digestibility of 100 units of feed dry matter will depend on the proportion of the different fractions present in the feed and their corresponding digestibilities. The cell content consisting of soluble carbohydrates, crude protein, ether extract and soluble ash have a potential digestibility close to 100% so in the model have been in-

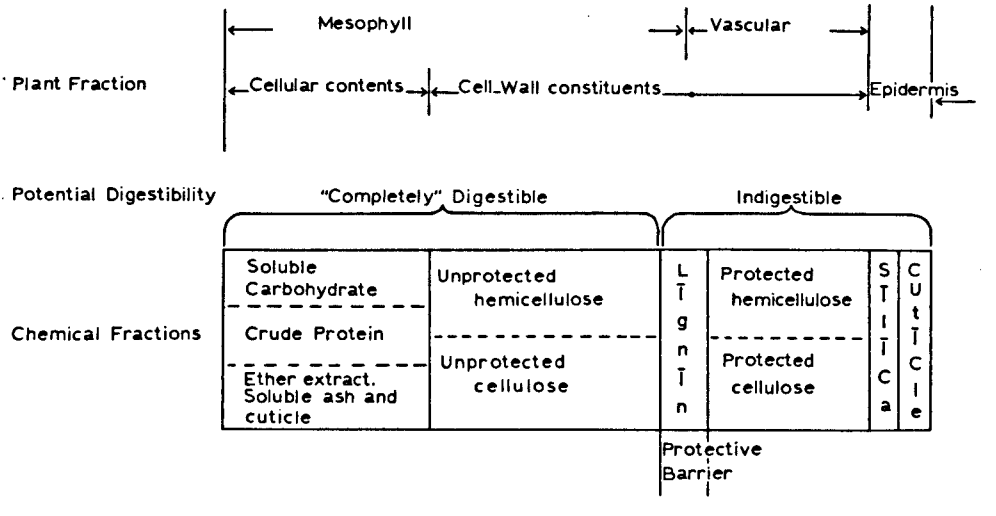

Figure 1. Model of the relation between plant and chemical fractions indicating areas of potential digestibility.

cluded in the 'completely digestible fraction' (Figure 1). At the opposite end of the model are the indigestible lignin, cuticle and silica.

The cellulose and hemicellulose are only partially digested and may therefore be considered as two fractions, a lignin encrusted fraction that is completely indigestible and a lignin-free unprotected fraction which has a potential digestibility of 100% (McLeod and Minson, 1974). Since the presence of lignin is the cause of the difference between the protected and unprotected cellulose and hemicellulose fractions, lignin is shown in the model as a barrier between potentially digestible and indigestible fractions of the cell wall (Figure 1). If digestible lignin were present in a feed it would not protect the cell wall from digestion and this lignin would not be included in the barrier section of the model.

The cuticle is an indigestible fraction which reduces the rate of digestion of the potentially digestible fraction of the plant (Monson and Burton, 1972). The cuticle is a surface layer and does not completely protect any potentially digestible fraction so it has been placed at the end of the summative model. However, it can be argued that since some cell wall is protected by a combination of both lignin and cuticle (Wilkins, 1969) then part of the cuticle should be included in the protective barrier part of the model.

The position of silica at the end of the summative model of potential digestibility is more debatable. A number of pasture species contain silica in specialized epidermal cells where it is unlikely to affect the digestion of other fractions. Sometimes silica is present as inclusions within the cell wall or as encrustations on the cell wall (Jones and Handrick, 1967) where it might influence the digestibility of the cell wall (Baker and Harriss, 1947). Using a regression approach, Van Soest and Jones (1968) showed that each percentage unit of silica reduced *in vivo* dry matter and organic matter digestibility by 3.0 and 1.4 percentage units respectively. Smith *et al.* (1971) showed that one unit of plant silica reduced the *in vitro* organic matter digestibility of forages by 0.98 units. By contrast silica was found to have no effect on the *in vivo* organic matter digestibility of Panicum (Minson, 1971) or legumes (Van Soest and Jones, 1968). These apparent differences could be explained by the position of silica within the plant tissue. It is also possible that other indigestible fractions may be found which protect the cell wall from digestion.

Changing potential digestibility

Increasing potential digestibility

The main factor controlling the potential digestibility of feed is the lignin encrustation on part of the cellulose and hemicellulose. Removal of this barrier by physical or chemical methods will increase the potential digestibility of the feed (Pigden and Bender, 1972). The lignin barrier may be ruptured by ball milling the forage. For immature grass this increased the cellulose digestibility from 85 to 93% but for mature grass the increase was from 46 to 91% (Dehority and Johnson, 1960). High velocity electrons

and gamma rays also destroy the structure of the cell wall and gamma rays have been used to increase in *in vitro* dry matter digestibility of wheat straw from 40% in the control to 95% in the treated material (PRITCHARD *et al.*, 1962). Alkali treatment has been widely used to improve the digestibility of roughages. The treatment enhances diffusion of liquids into the cell wall and makes the cellulose and hemicellulose more accessible to the bacterial enzymes (SMITH *et al.*, 1970).

Decreasing potential digestibility

As pastures mature there is an increase in the proportion of cell wall protected by lignin and hence a fall in dry matter digestibility (RAYMOND, 1969). Although dry matter digestibility is correlated with lignin percentage of feed, the depressing effect on digestibility of each unit of lignin varies between forages (TOMLIN *et al.*, 1965). This lack of uniformity in response is to be expected because it is unlikely that lignin would be laid down in such manner that one unit of lignin always protects the same quantity of hemicellulose and cellulose in different plant species, plant parts or at different stages of growth.

Reduction in the level of soluble carbohydrate in pasture occurs at night (SMITH, 1973) and during natural drying for hay (KERR and BROWN, 1965). These losses will automatically lead to an increase in the proportion of all the other cellular constituents including the protected cell wall and hence a reduction in the potential digestibility of the feed. Although in these instances a decrease in the soluble carbohydrates leads directly to a fall in potential digestibility it is always possible to have plants with the same potential digestibility yet different levels of soluble carbohydrates. Similarly the protein content may be changed without altering digestibility since the increase in protein is often offset by a depression in soluble carbohydrates (ALBERDA, 1965).

The digestibility of protein in forages may be reduced by high temperature drying (DIJKSTRA, 1954) and treatment with tannin (MCLEOD, 1974) or formaldehyde (HEMSLEY *et al.*, 1970). The extent of the decrease in digestibility depends on the duration and concentration of the treatments.

LOSS OF POTENTIALLY DIGESTIBLE MATERIAL

In 1942 MCANALLY demonstrated that the digestive system is not 100% efficient in removing all the potentially digestible nutrients from a feed and in the case of sheep fed with straw he found that 12% of the cellulose excreted in the faeces was potentially digestible. Subsequent work by WILKINS (1969) confirmed that this principle applied to a range of tropical and temperate grasses at different stages of growth. The extent of the loss of potentially digestible material depends on many animal and dietary factors and these will now be considered. In all the examples discussed only *difference* in digestive efficiency will be cited since no data has yet been published on losses of potentially digestible material.

Rumination

Rumination increases the surface area available for microbial action in the rumen and voluntary food intake by reducing the time feed is retained in the rumen (PEARCE and MOIR, 1964). By using muzzles fitted to sheep between meals PEARCE and MOIR (1964) showed that rumination of a chaffed oaten hay/lucerne diet reduced the time feed was retained in the digestive tract by 66% causing a *decrease* in dry matter digestibility from 77.4 to 70.8%. Similarly, a reduction was found when rumination was stimulated in sheep by adding polyethelene flakes to a ground diet on which there was little natural rumination (PEARCE and MOIR, 1964). The main cause of this decrease in digestibility with rumination was a drop in the digestibility of the crude fibre fraction although there was also a decrease in the apparent protein digestibility indicating either a decrease in the true digestibility of the protein or an increase in the level of metabolic excretion.

Fineness of grinding

The effect of grinding on digestive efficiency is similar to that caused by rumination. When sheep were fed hay ground through screens of different size (12.7 mm, 4.8 mm, 3.1 mm and 1.0 mm) ALWASH and THOMAS (1974) found that hay with the small particle size was retained for a shorter time in the digestive tract and the sheep were less efficient at digesting the organic matter, crude fibre and protein. The experiment was carried out at two levels of feeding (1.1 and 2.4 times maintenance) with maximum depression in organic matter digestibility of 6.6 and 7.5% respectively at the higher feeding levels.

Level of feeding

Increasing the level of feed intake causes a decrease in the digestive efficiency of sheep for both organic matter and crude protein (RAYMOND *et al.*, 1959). ALWASH and THOMAS (1971) found a large interaction between level of feeding and feed composition. When level of intake was increased from 1 to 3.1 times maintenance digestive efficiency of sheep fed a chopped diet was reduced by 1.8% compared with 9.8% for the same feed ground and pelleted. The corresponding decreases in the apparent digestibility of the crude protein were 4.2% and 5.4% respectively and for the crude fibre 2.4% and 17.8% respectively. These decreases in digestive efficiency were associated with a reduction in the mean time feed was retained in the digestive tract. In another study ALWASH and THOMAS (1971) confirmed the effect of retention time on digestive efficiency but also found that with ground grass fed at high levels there was a decrease in pH and rate of breakdown of cotton thread in the rumen and suggested that 'the completeness of digestion also depends on the intensity of the digestive processes'. Recently TYRRELL and MOE (1975) demonstrated that the size of the depression in digestive efficiency caused by increased level of intake was related to the proportion of grain in the diet. With high grain diets increasing the level of intake caused large decreases in digestive efficiency but with low grain diets only small decreases were observed.

Animal species

BLAXTER and WAINMAN (1964) found that cattle digested hay more efficiently than sheep but on a maize based diet the sheep digested the feed more efficiently than cattle. The maximum difference between sheep and cattle in energy digestibility was 4.2%. In other comparisons using oat straw, hay and dried grass, sheep were less efficient than steers by 7.6, 2.8 and 5.3% respectively. Similar differences have been reported for a tropical grass (PLAYNE, 1970). The lower digestive efficiency of sheep for roughages has recently been shown to be associated with a much shorter retention time of feed in the reticulo-rumen (M. C. REES and D. A. LITTLE, unpublished data).

The potential digestibility of the feeds used in these studies was not measured but the existence of such large differences between sheep and cattle illustrates that larger losses of potentially digestible material occur in sheep than in cattle.

Effect of intestinal parasites

SPEDDING (1954, 1955) has shown that intestinal parasites reduce the efficiency with which sheep digest organic matter by 1.6 and 1.4%. The apparent digestibility of the crude protein was also decreased, resulting in an additional excretion of 0.50 and 0.54 g crude protein per 100 g of feed. Thus one third of the depressing effect of worms on digestive efficiency can be accounted for by the decrease in the apparent digestibility of protein.

Essential nutrients

Rumen bacteria require a supply of protein, amino acids or their precursors, non-protein nitrogen and sulphur (HUNGATE, 1966). Urea has been found to increase the dry matter digestibility of low nitrogen roughage fed to sheep (HARRIS and MITCHELL 1941, GRAHAM 1966) and cows (CAMPLING *et al.*, 1962). This rise in digestibility indicates that a nitrogen deficiency in the diet may reduce the efficiency with which animals digest the potentially digestible fraction of the diet. Supplementing sulphur deficient diets with sulphur will also increase digestibility. Recently REES *et al.* (1974) increased the dry matter digestibility of *Digitaria decumbens* 5% by adding sulphur and showed that this rise in digestibility occurred despite a reduction in the time feed was retained in the rumen.

UNAVOIDABLE METABOLIC FAECAL LOSS

VAN SOEST (1967) estimated the endogenous excretion of dry matter amounts to 12.9% of the dry matter eaten. Thus the *maximum* apparent *in vivo* digestibility would be the potential digestibility percentage of the feed minus 12.9. However, metabolic faecal loss is not constant under all conditions. COMBELLAS *et al.* (1971) suggests a value of 10.7% and Colburn *et* al. (1968) 9.8%. When expressed on an ash free basis metabolic faecal loss was 9.5% (MINSON 1971).

PREDICTING APPARENT DIGESTIBILITY

The relation between digestibility and feed composition may be studied either empirically or factorially. Empirical methods rely on regressions relating digestibility to one of more fractions of the feed determined by chemical or bioassay methods. Although these empirical methods provide sufficiently reliable results for farm advisory work and plant breeding and selection programmes, they have many limitations. Their use with feeds other than those used to derive the regressions can lead to large errors in predicting digestibility, while regressions derived with sheep obviously cannot be applied to cattle which digest their feed more efficiently. This problem may be overcome by expanding the biological base of the data used to derive the regression, but only at the expense of increasing the error that must be applied to any prediction of digestibility (MINSON and KEMP, 1961). Thus there is a serious limitation to the accuracy that can ever be achieved when using empirical methods.

An alternative approach to estimating digestibility is to acquire 'a fundamental knowledge of the constituents which influence the digestibility of a roughage and of their resistance and interaction during the digestion of the food by the rumen microorganisms since this may lead to a chemical analysis from which the apparent *in vivo* digestibility of the organic matter can be calculated more accurately' (GAILLARD, 1966). This factorial approach to digestibility relies on isolating fractions of constant digestibility or fractions which vary in digestibility in some predictable way. Many chemical methods have been proposed for separating the readily digested cell contents from the cell wall and for determining the indigestible lignin in the cell wall (CRAMPTON and MAYNARD, 1938; GAILLARD, 1958, 1962; JARRIGE and MINSON, 1964; WAITE and GORROD, 1959; VAN SOEST, 1967). The main problem has been to determine the digestibility of the cell wall; regressions relating cell wall digestibility to the lignin content of acid detergent fibre (VAN SOEST and MOORE, 1965) or the ratio of lignin to the content of cellulose + hemicellulose (MINSON, 1971) have been used. All these chemical methods of estimating the digestibility of the cell wall are emperical and are unrelated to the histological observation that some cell walls are completely degraded, whereas cell walls protected with lignin and cuticle are indigestible (WILKINS, 1969). It is therefore logical to subdivide the chemically determined cell wall constituents into two fractions according to whether or not they are encrusted with lignin as indicated in the summative model of potential digestibility (Figure 1).

The best available method to divide the cell wall into a protected and unprotected fraction is prolonged digestion (WILKINS, 1969). Histological studies can be helpful (Pigden, 1953) but from an examination of cross sections of the leaf or stem it is difficult to decide in all cases whether or not a particular piece of cell wall is completely protected. A major advantage of the prolonged digestion method of dividing the cell wall into two fractions is that it automatically includes any protective effect of silica or cuticle.

The efficient digestion of feed by animals was shown to be related to many animal

factors such as species, level of intake and intestinal parasites but there were interactions with the feed. Where the interactions are caused by a deficiency of nitrogen or sulphur required by the rumen microorganisms it might be possible to predict the depression in digestive efficiency by chemical analysis of the feed but this would also require a knowledge of S and N status of the animal used. Depressions in digestive efficiency can also be caused by physical factors in the diet. Any attempt to estimate the expected loss of potentially digestible material by an animal would therefore require a knowledge of both chemical and physical composition of the feed and the way these react with animals with different levels of nutrient reserves, requirement and abilities to recycle essential nutrients.

When the digestive efficiency of the animal was reduced by increased level of feeding, intestinal parasites of physical form of the diet, there was a decrease in the time feed was retained in the digestive tract. This reduction would limit the time for bacterial breakdown of the cell wall and account for the observed depressions in digestibility of the cell wall. According to the results in Table 1 the crude protein in plants has a true digestibility of about 90 % so would not be expected to vary with different feeding conditions. Rather surprisingly the apparent digestibility of crude protein was always reduced when there was a reduction in retention time and a depression in cell wall digestibility. These results casts doubts on the assumption that the digestibility of the cell contents is independent of the digestibility of the cell wall.

Conclusion

The *in vivo* digestibility of a feed depends on the chemical and physical composition of the diet and the efficiency with which the animal digests the potentially digestible fraction of the feed. Many factors are involved and so little is known about their interactions that it appears more desirable to have a simple theoretical model of potential digestibility and to describe the many factors causing deviations than to attempt to develop one comprehensive regression including all factors. This simple model should lead to a better understanding of the plant and animal factors controlling the apparent digestibility of feeds.

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MICROBIOLOGICAL ASPECTS OF PLANT CELL WALL DIGESTION IN THE RUMEN AND CECUM: SOME RECENT DEVELOPMENTS †

R. A. PRINS*

Laboratory of Veterinary Biochemistry, State University of Utrecht, Utrecht, The Netherlands

All vertebrate herbivores as well as many invertebrates feeding on plants depend on their gut microbes for the utilization of the various carbohydrate polymers present in the plant cell wall. Polymers of β-glucose (cellulose, lichenin), of galacturonic acid (pectins) and pentoses (hemicelluloses) cannot be hydrolyzed by enzymes of the host animal and only some invertebrates have been found to produce cellulases of their own.

This type of symbiosis (mutualism) between microorganisms and herbivorous hosts has been a very succesful development as witnessed by the large number of animal species from several classes of the animal kingdom with this type of association.

With mammalian species in general two types of gastrointestinal adaptations are found to harbour a large microbial population, allowing it to act on the plant tissues eaten. In a number of herbivorous mammals there are dilatations of the oesophagus or *diverticula* of the simple stomach to form a rumen or rumen-like stomach(s), whereas herbivores lacking such a forestomach invariably have been found to possess an enlarged cecum or colon (MCBEE, 1971). The development of such devices to accommodate the bulky vegetable food for a prolonged incubation in the fermentative chambers has enabled the colonization of nutritionally poor or even toxic environments (MOIR, 1968).

Most of our knowledge concerning the microbiological aspects of the digestion of plant material in the gastrointestinal tract of mammals has been obtained from studies on ruminants or on the microorganisms isolated from their forestomachs. The contents of the plant cells in forage which contain the cytoplasmatic proteins, storage polysaccharides, pectins, sugars, lipids and nonprotein compounds are very digestible to ruminants (VAN SOEST, 1967). Even the digestibility of mature woody twigs is highly correlated with the cell contents expressed as percent dry matter (SHORT *et al.*, 1974). The digestion of the cell wall components, however, is relatively slow (ALEXANDER *et al.*, 1969a) and varies among forages. Plants with high cell wall component levels, especially when combined with high lignin contents, are low-quality forage. In determining the digestibility of cell walls the lignin: cellulose ratio appears to be more

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† This is a report.

closely related to digestibility than to any other measurement (VAN SOEST and MCQUEEN, 1973).

In recent years several studies have been undertaken into the physical interrelationships of rumen bacteria to various plant tissues during digestion. Leaf sections of grasses and other forages were incubated with rumen contents and the digestion process studied by light microscopy (HANNA *et al.*, 1973; KAWAMURA *et al.*, 1975), or by scanning and transmission electron microscopy (AKIN *et al.*, 1973, 1974; AKIN and AMOS, 1975). All authors agree that the non-lignified mesophyll and phloem are degraded prior to all other tissues in forage. In many cases significant degradation of these tissues occurs within 12 hours of incubation without bacterial attachment to the plant cells and it is likely therefore that extracellular enzymes are adsorbed on the cell wall surface prior to digestion. There is evidence (see AKIN *et al.*, 1974), that the microfibrillar network of cellulose in these primary cell walls is loose, with the fibrils having a low and non-uniform degree of polymerization. Penetration and degradation of such structures by extracellular enzymes appears relatively easy. The rate of digestion of cell wall material from many of the common vegetables is very rapid, more than 90% being digested with 15 hours and the digestion of this type of fiber may be important in nonruminants (reviewed by MCQUEEN and VAN SOEST, 1973). It is likely that much of the cellulose in the mesophyll and phloem of vegetables is of the type described above.

From the studies by Akin and his associates it became clear that thick-walled cells present in the epidermis and bundle sheath are degraded more slowly and often not before bacteria are attached to the plant cells. The attachment sometimes appears to be mediated by a dense extracellular matrix, that firmly binds the microorganisms to the plant cell walls (Fig. 1a), but in other instances these coats are thin of the association between bacteria and plant cell walls may even be so close, that the points of juncture become indistinguishable (Fig. 1b).

The aspect of microbial attachment to the insoluble substrate cellulose has been studied by PATTERSON *et al.* (1975). *Ruminococcus albus*, a cellulolytic rumen coccus produces a polysaccharide 'coat' layer external to the outer membrane. This layer stains specifically with rutenium red and consists of a compact mat of fibers adjecent to the cell, that mediate the attachment of the cells to cellulose when this substrate is added to the pure cultures. Not only in pure culture, but also in natural rumen contents all gram-negative cells can be seen to possess some kind of surface structure (CHENG and COSTERTON, 1975). Ten different morphological variants of an external structure were discerned. Similar observations have been made with respect to the morphology of bacteria from other habitats such as marine and freshwater environments, soil and infected tissue. In all these environments the external structures probably serve as a means for attachment of the cells to their insoluble substrates, allowing a close contact before their degradative enzymes can act. The whole cell envelope functions also in the protection of the cells (COSTERTON *et al.*, 1974) and in the retention of wall-associated hydrolytic enzymes which are bound to structural elements in the peri-

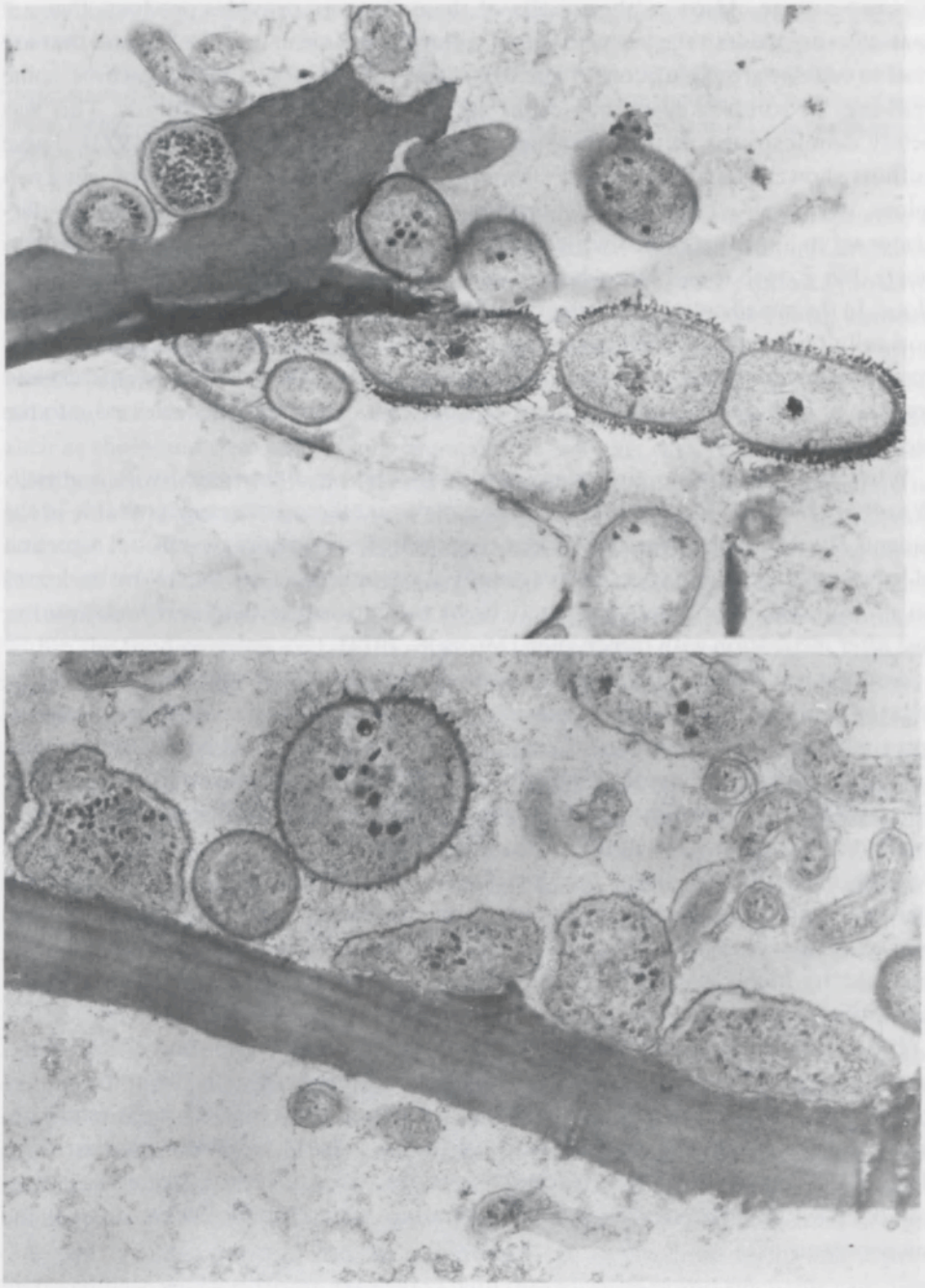

Fig. 1a, b. Rumen bacterial attachment and degradation of thick-walled bundle sheath cells after incubation. Some bacteria appear to attach to the plant cell wall by means of an extracellular substance (Fig. 1a), while other bacteria maintain close association to the plant cell wall without visible evidence of such a substance (Fig. 1b). Magnifications: Fig. 1a 36,000 ×; Fig. 1b 39,100 ×. Photographs by Dr. D. E. AKIN.

plasmatic space. Also: ...'the activity of these enzymes provides products that are spatially very close to the permeases that will transport them into the cell and that are vital to cellular growth' (quoted from CHENG and COSTERTON, 1975). However, some spill-over of soluble sugars may occur during polysaccharide hydrolysis. This was neatly demonstrated in the experiments by SCHEIFINGER and WOLIN (1973). These authors showed that *Selenomonas ruminantium*, an organism incapable of using cellulose, grew well when cultured together with the cellulolytic rod *Bacteroides succinogenes* in a synthetic medium containing ball-milled filter paper as the only substrate. No growth took place when this medium was inoculated with *S. ruminantium* alone. In the mixed cultures the sugars released by enzyme action are intercepted by the non-cellulolytic strain. The importance of this spill-over in the rumen should be investigated more closely using gnotobiotic mixed cultures. It would be of interest and realistic to include proteolytic strains that could act on the enzymes released into the medium.

Where it is exposed to air, the epidermis of plants is often coated with a cuticle. Waxes are the primary constituent of the cuticle and these are non-degradable in the rumen. Thus, the cuticle forms a barrier to entry by the microorganisms, but digestion of leaves or other plant tissues occurs readily at cut or broken surfaces. Pressing leaves on a rough surface or abrading them to break the cuticle increased *in vitro* dry matter loss after incubation with rumen fluid (MONSON, 1973).

Another result of the microsopical studies by AKIN *et al.* (1974) was the observation that the propensity for bacterial attachment to highly lignified cells of vascular tissue (protophloem fiber, secondary xylem, and sclerenchyma) is low and that these cells are not significantly degraded even after 70–90 hours of incubation with rumen fluid. Often, these cells have little or no contents and thus their nutritional value is low. While these microscopial studies demonstrated that microbial digestion of plants depends on the relative amounts of leaf made up by mesophyll, phloem, xylem, cutinized walls and other anatomical structures, they also make clear that the steric disposition of the tissues is of importance. When, e.g. highly degradable phloem of grasses is enclosed by highly lignified tissues, the rate of disintegration will be lowered appreciably.

The cavities which are formed upon bacterial breakdown of the more resistant plant structures are often lined with microorganisms as can be seen in the beautiful photographs made by AKIN and his coworkers. These zones of erosion were already observed by BAKER and MARTIN (1938). CZERKAWSKI (1969) has pointed out that when the acidic fermentation products formed from the cell wall carbohydrates would accumulate within these cavities, their concentrations would rapidly become toxic to the microorganisms:...

'Consider such an eroded cavity, roughly spherical and with a diameter of about 10 μ, i.e., corresponding to about 10–20 diameters of cellulolytic bacteria. The volume of such a hole is 5×10^{-10} cm^3, and if one-fifth of the space of the hole was originally occupied by cellulose, the dissimilated carbohydrate in solution inside the cavity would amount to approximately 10^{-10} g. If each mole of

simple sugar gives 3 moles of products (HUNGATE, 1966) ranging from formic to butyric acids, the hole will contain $3/180 \times 10^{-10}$ moles of these products. This would give a concentration of about 3 M. The concentration of the steam volatile fatty acids in the rumen is about 0.1 M and it is unlikely that the cellulose digesters would survive or at least continue fermentation in a 3 M solution of acids unless there was an efficient provision for removal of such products and conversion to less damaging ones, such as, for instance, methane', (quoted from CZERKAWSKI, 1969).

It is unlikely that such high concentrations of products would accumulate since a) some of the primary fermentation products (succinate, formate, lactate) are intermediates in the mixed rumen cultures and are converted to other products (however most of these products are still acids) and b) constant mixing of the rumen contents occurs in the normal animal and will help to prevent the build up of high concentrations of fermentation products. Recent results, however, indicate that both of these processes are inadequate to prevent some accumulation and that even in ruminants such as sheep and deer with homogenous rumen contents with respect to dry matter distribution, gradients of fermentation products exist at the surface of plant material in the rumen digesta (PRINS and VAN HOVEN, 1976a). Several workers (e.g. ALEXANDER *et al.*, 1969b; CZERKAWSKY and BRECKENRIDGE. 1969) have found that the distribution of the soluble marker polyethlene glycol (PEG) in the total water in the rumen is not uniform, but that PEG is excluded from a large portion of the water contained within the food particles and microorganisms. Since the water in the solids is poorly equilibrated with the remainder of the rumen liquid phase, high concentrations of primary fermentation products may accumulate in this small fluid layer. The results obtained by PRINS and VAN HOVEN (1976a) indeed showed that concentrations of lactate, succinate and acetate were much higher in the liquid phase associated with the fibrous material in the rumen than in the liquid phase obtained by filtering rumen contents through gauze. Preliminary experiments with mixed rumen organisms indicate that assuming a pH of 6.7 at the particle surface, the observed concentration difference of acetate in its own would cause a 15% decrease of the fermentation rate. With lower pH the inhibition becomes more severe, since the undissociated acids pass rapidly through the bacterial membranes.

The cellulolytic rumen bacteria are not all equally effective in hydrolyzing forage cellulose. Thus, *Bacteroides succinogenes* and *Ruminococcus* species digest more of the cellulose or dry matter in forages than *Butyrivibrio fibrisolvens* (Table 1). Some idea about the characteristics of the bacterial cellulases can already be obtained from the extent and shape of the clearing zones which are formed around colonies of cellulolytic bacteria grown in cellulose-agar media (HUNGATE, 1966). The cellulose in such media around colonies of *B. fibrisolvens* is not always cleared to the same extent as around colonies of *B. succinogenes* and the ruminococci. *B. succinogenes* forms an extracellular cellulase which is firmly associated with the cell surface and since this organism does not form distinct colonies but migrates through the agar, irregularly formed clearing areas are produced in agar media. *Ruminococcus* sp. form distinct colonies on cellulose-agar which are often surrounded by clear zones of cellulolysis. This can be

Table 1. Extent of cellulose digestibility in various forages by pure cultures of cellulolytic rumen bacteria.[a]

Substrate	Incubation time	Organisms			
		B. succinogenes	*R. albus*	*R. flavefaciens*	*B. fibrisolvens*
Alfalfa (prebloom)	1 week	62–64 (2)[b]	53 (1)	28–54 (4)	6 (1)
Brome grass (boot stage)	1 week	79–81 (2)	57 (2)	48–65 (4)	15 (1)
Teff hay	3 weeks		43–56 (2)	38–66 (2)	10–37 (10)

[a] Data for alfalfa and brome grass from DEHORITY and SCOTT (1967) and data for teff hay from KOCK and KISTNER (1969).

[b] Percent of cellulose digested; the number between brackets indicates the number of strains used.

taken as evidence for a diffusable cellulase that becomes firmly bound to the substrate and that will only move on after the substrate has been digested. Part of the cellulase activity of *R. albus* is destroyed upon exposure to oxygen and the enzyme is inhibited by cellobiose (SMITH *et al.*, 1973). This is in accordance with the observations that when the cellulose-agar media, in which *R. albus* (FUSEE and LEATHERWOOD, 1972) of *B. fibrisolvens* (HUNGATE, 1966) were grown, contains cellobiose, the cellulose is not cleared around the colonies until the sugar has been used, suggesting feedback control.

B. fibrisolvens can use many carbohydrates for growth and since growth on soluble carbohydrates is usually much faster than on cellulose or other insoluble polysaccharides, it is doubtful whether this organism is very important for the breakdown of cell wall polysaccharides in the rumen. It is the predominant culturable cellulolytic

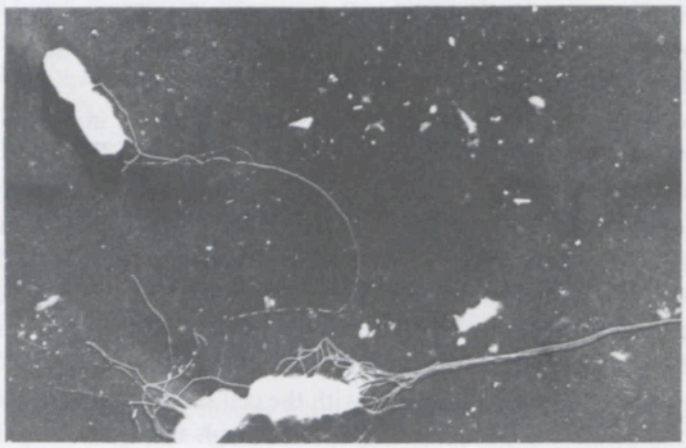

Fig. 2. Electron micrograph of cells of *Eubacterium cellulosolvens* strain 271 isolated from the bovine rumen (see PRINS *et al.* 1972). Magnification: 12,400 ×.

organism from the rumen of deer (McBee *et al.*, 1969; Prins, unpublished experiments) which have a limited capacity to digest cellulose (Prins and Geelen, 1971). The organism *Eubacterium cellulosolvens* (Fig. 2) shares many characteristics of the butyrivibrios (motility, fermentation products, capacity to use many sugars), yet clearings in celluloseagar are distinct (Fig. 3). In the rumen of dairy cattle on a diet of hay and concentrates *Eub. cellulosolvens* occurred in high numbers and comprised at least 50% of the total cellulolytic count (Prins *et al.*, 1972).

Some of the cellulolytic bacteria mentioned above are also among the predominant hemicellulose digesters in the rumen. A number of studies with pure cultures have been carried out by Dehority and coworkers, which have given much interesting information about the degradation and utilization of both isolated hemicellulose and hemicellulose in intact forages (see review by Dehority, 1973). The fermentation of both isolated and intact forage hemicelluloses appears to occur in two stages. The first involves a solubilization or depolymerization of the hemicellulose into 80% ethanol-soluble oligosaccharides. In the second stage these oligosaccharides are fermented. Upon breakdown of hemicelluloses xylose- and arabinose-containing oligosaccharides accumulate in the medium (but only temporarily, when they are subsequently utilized), but little or no free xylose and/or arabinose appear. In fact, utilizing strains may even lack the capacity to ferment xylose and arabinose (Dehority, 1967).

Whereas only those strains of cellulolytic and non-cellulolytic rumen bacteria capable of growth on xylan could degrade as well as subsequently utilize hemicelluloses

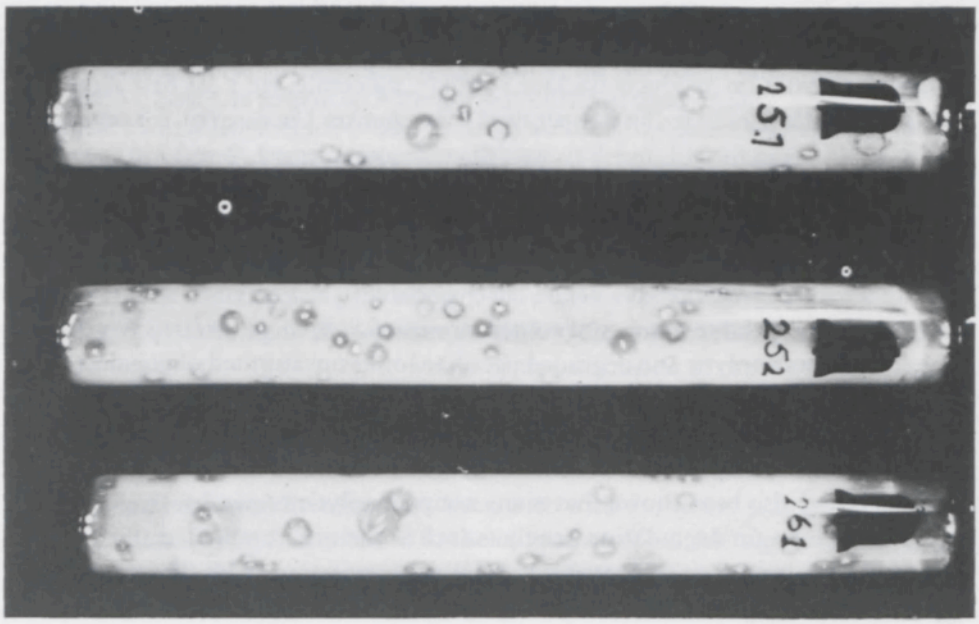

Fig. 3. Colonies of three strains of *Eubacterium cellulosolvens* in rumen fluid cellulose agar. Darker colored zones around colonies indicate cellulolysis. Actual size.

for growth, degradation without utilization was observed upon incubation of hemicelluloses with xylan-negative cellulolytic strains. In the latter case the degradation was thought to be the result of a non-specific cleavage of $\beta 1 \rightarrow 4$ xylosidic linkages by cellulases. The extent of degradation was not related to the capacity of the organism to utilize the substrate. The extent of degradation was also not related to the overall sugar composition of the hemicelluloses:

> ....'while chemical data relate compositional differences of forages to digestibility, these do not fully explain digestibility variances. Investigations with gnotobiotic fermentations have indicated that the molecular orientation and complexity of certain cell wall constituent may affect forage degradation by rumen microbes...' (quoted from COEN and DEHORITY, 1970).

It was already known from the work of GAILLARD *et al.* (1965) and BAILEY and GAILLARD (1965) that hemicellulose structure is a decisive factor in controlling digestibility.

Much more complicated situations can be visualized than the simple two-step cooperation between degraders and utilizers described above. Some possibilities are schematically drawn in Fig. 4. In this figure case (a) represents the situation in which only one organism is involved in the degradation of the polymer as well as in the subsequent fermentation of the oligomers; in case (b) a second organism (or strain of the same species) uses the bulk of the oligomer(s) produced by organism 1;in case (c) the enzyme(s) of organism 2 are required to make certain lower oligomers available to organism 1, while in cases (d) and (e) a third or fourth organism uses the products liberated by organisms 1 and 2 in concerted action, etc. One or more of the organisms 1–4 and additional types 5, 6, etc. may also be involved in the conversion of each others primary fermentation products (lactate, formate, succinate, etc.). At first sight there seems to be little benefit in this cooperation for organism 1 in case (b), for organism 2 in case (c), for organisms 1 and 2 in case (d) or for organisms 1, 2 and 3 in case (e). It could be, however, that such organisms use their degradative power only to hydrolyze certain cell wall constituents in order to reach other polymers which they can ferment, or more directly, to release minor carbohydrate constituents which are part of the polymer structure. An example of the latter possibility can be given with respect to pectin digestion. A large number of rumen strains of *Streptococcus bovis* were shown to be actively pectinolytic and degraded pectin to lower unsaturated oligogalacturonides (ZIOLECKI *et al.*, 1972), indicating true pectin lyase activity. However, none of these pectin-hydrolyzing strains could utilize the oligogalacturonides produced nor galacturonic acid, despite the fact that some of these strains were isolated on a pectin medium. It had also been shown that many nonpectinolytic rumen bacteria of several genera can use pectin degradation products such as the unsaturated tetramer, trimer, dimer and galacturonic acid (TOMERSKA and WOJCIECHOWICZ, 1973). The degradation of galacturonide oligomers is caused by highly specific intracellular microbial enzymes, which can be distinguished from pectin depolymerases by the fact that the rate of substrate breakdown is inversely proportional to the chain length of the substrate (ROM-

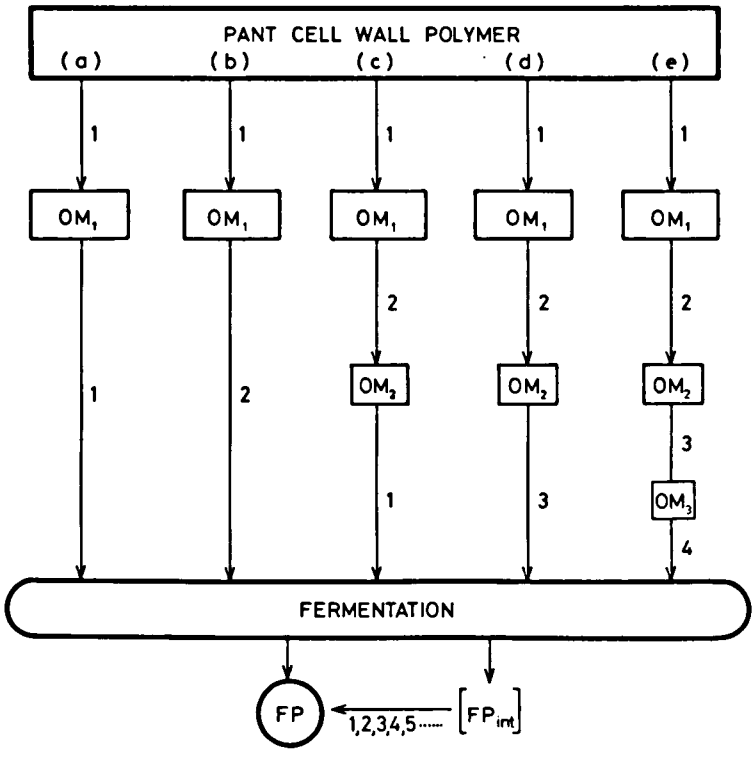

Fig. 4. Schematical representation showing examples of possibilities for cooperation between rumen microorganisms in the lysis and subsequent fermentation of plant cell wall polymers.
OM_1, OM_2, OM_3: lytic products (oligomers) produced from the polymerous substrate(s) by enzymes of organisms 1, 2 and 3. In general OM_3 will be of simpler structure than OM_2, etc.
FP_{int}: those primary fermentation products (lactate, succinate, formate, hydrogen) than can still be converted in the rumen, thus becoming intermediates.
FP: pool of fermentation end-products.

BOUTS, 1972). The cooperation between pectinolytic strains of *S. bovis* and nonpectinolytic rumen bacteria using the oligogalacturonides constitutes an example of case (b) in Fig. 4. According to ZIOLECKI *et al.* (1972), the main biological significance of the pectinolytic activity of *S. bovis* would be the utilization of carbohydrates that always occur in natural pectins and which are released in the course of pectin breakdown. Several of these sugars (arabinose, xylose, galactose, rhamnose) can be used by *S. bovis* for growth.

A seemingly similar situation has been met with *Isotrichia prostoma*, a rumen holotrich ciliate which is specialized in the utilization of soluble sugars in the rumen. When intact cells of *I. prostoma* are incubated with apple pectin the rate of hydrogen evolution as well as the rate of amylopectin storage within the cells, are increased appreciably above the control without substrate (PRINS and VAN HOVEN, 1976b). Thus, the organism is able to use certain compounds ligerated from pectin by its enzymes.

Much lower activities were observed with polygalacturonic acid, and almost no activity with a mixture of oligogalacturonides, with tetragalacturonic acid or with galacturonic acid. This is remarkable since both a pectinesterase and a polygalacturonase has been described for *Isotrichia prostoma* (ABOU AKKADA and HOWARD, 1961). Recent studies have shown, however, that there is no polygalacturonase in *Isotrichia prostoma* but the enzyme is a pectate lyase producing the unsaturated trimer and tetramer (ROMBOUTS and PRINS, unpublished data) and these could be the products that are fermented. The uptake of polygalacturonic acid in the protozoa could be difficult since it is a charged molecule.

Most interesting results obtained by DEHORITY and coworkers were those which clearly indicated synergism between rumen bacteria. When hemicellulose-degrading strains (but non-utilizing) were combined with strains that can utilize the degradation products (but are non-degrading), more of the substrate hemicellulose was used. Similar observations have been made with regard to pectin degradation (GRADEL and DEHORITY, 1972) and even with regard to cellulose degradation, although in the latter case not of the same magnitude as with the hemicelluloses (DEHORITY and SCOTT, 1967). Synergism has always been assumed to be of importance in mixed cultures such as the rumen, but was lacking experimental evidence. While most of the results on synergism studies were obtained from experiments in which the extent of degradation was measured, the authors agree that it would be of major interest to study digestion rates of cell wall polymers under similar conditions.

As already mentioned *Isotrichia* (and also *Dasytrichia*) species belong to the so-called holotrich rumen ciliates. Another group of ciliate protozoa occurring in the rumen are the entodiniomorphs which are chiefly concerned with the digestion of plant particulate matter; it can be safely assumed that this group is of more importance for the digestion of cell wall polysaccharides than the holotrichs. Hemicellulase and cellulase activity have been detected in entodiniomorphs but not in holotrichs (reviewed by ABOU AKKADA, 1965; HUNGATE, 1966 and HARMEYER, 1973). Many entodiniomorphs ingest cellulose-containing plant particles and digest these at least partially, whereas the holotrichs do not. In the cecum, at least the protozoa are different from the species occurring in the rumen and *Cycloposthium* species from the cecum and colon of the horse are most frequently seen to have ingested fibrous material (Fig. 5). Cell-free extracts of these protozoa hydrolyze cellulose powder with the production of glucose and cellobiose, while cell-free cecal fluid had negligible cellulolytic activity (BONHOMME-FLORENTIN, 1969).

The digestion of the more refreactory cell wall polymers is a slow proces, since it takes time for the bacteria to attach to the cell wall and the enzymatic digestion of the polymers may be hindered by the presence of encrusting materials such as lignin. When the content of soluble lignin in the maturing plant decreases, the insoluble lignin associated with the cell wall increases and largely determines dry matter digestibility. With a lignin content of nearly 40%, the digestibility may become zero. Several investigators have shown that the accessibility of cell wall polysaccharides to (rumen) mi-

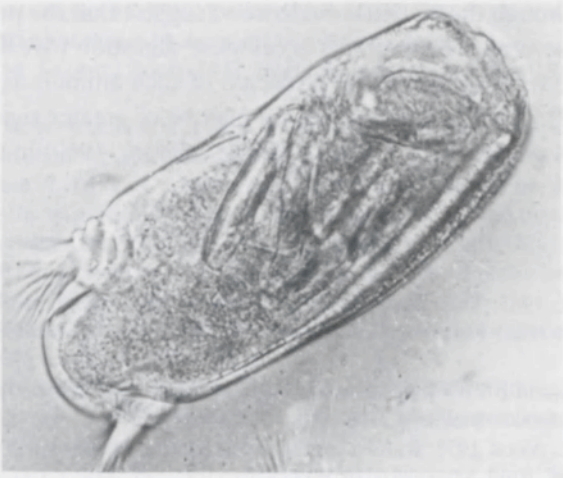

Fig. 5. Ciliate of the genus *Cycloposthium* from the horse cecum with ingested plant fibers. Photograph by Dr. A. BONHOMME-FLORENTIN.

croorganisms and their enzymes is increased upon delignification.

When the fermentation rate is low, microbial growth rates are low. It is known that when the growth rate of a microbe increases, growth of the organisms becomes more efficient, since the energy expended in maintenance becomes a smaller fraction of the total ATP made available during oxidation of the substrate (STOUTHAMER and BETTENHAUSEN, 1973). The energy level of the feed, while limiting growth, has little effect on the efficiency of cell production, but with increasing growth rates there will be a greater efficiency of cell production (ISAACSON *et al.*, 1975). These workers conclude that within the physiological range of growth rates encountered in the rumen, the maintenance energy has a dramatic effect on cell yield. The maintenance requirement, therefore, becomes a protein cost to the animal and not necessarily an energy cost, since volatile fatty acids are produced at the same rate. It can be expected that in the rumen of small ruminants which have a limited capacity to digest cellulose (PRINS and GEELEN, 1971; SCHOONVELD *et al.*, 1974) and which select for more nutritious, easily digestible forages, growth of the microbes will be more efficient per mole of digestible substrate, since rates of microbial fermentation and growth and the rate of passage of ingesta will all be higher than in large ruminants.

Feeding diets with low digestibility will increase rumen retention times, slow down the rate of passage of ingesta and will have a negative influence on feed intake (BAILE and FORBES, 1974). Under conditions where the turnover of rumen solids is slow, most if not all of the digestible fiber will be fermented in the rumen and little fermentation of cellulose and hemicellulose will take place in the lower gut (GAILLARD and VAN 'T KLOOSTER, 1969). When, on the other hand, for some reason (e.g. as a result of grinding of the feed) the rate of passage is increased, more digestible material will flow out of the rumen and then the cecum may play a more important role as a site for micro-

bial digestion. Although there is little evidence to suggest that the primary function of the cecal flora in several nonruminants is cellulose digestion (McBee, 1971), 30% of the cellulose in the feed is digested in the cecum of such animals as beavers (Hoover and Clarke, 1972). Hemicellulose digestion may be of greater significance than cellulose digestion (Keys and Van Soest, 1970; Keys *et al.*, 1969) in the cecum of nonruminants.

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SEARCH FOR FACTORS OTHER THAN 'LIGNIN-SHIELDING' IN PROTECTION OF CELL-WALL POLYSACCHARIDES FROM DIGESTION IN THE RUMEN*

by G. N. RICHARDS

Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville, Australia

INTRODUCTION

The major components by weight of the diet of grazing ruminants are plant cell-walls. These comprise the major energy source of the animals and they are composed mainly of celluloses hemicelluloses and pectic substances together with 5–20% of lignin. In the rumen the polysaccharide constituents are only partly digested (e.g. half of the total polysaccharide component disappears from the fibrous digesta and is presumed 'digested'). The long accepted view of this resistance to digestion of part of the cell-wall polysaccharides is that physical protection by lignin prevents attack by the rumen microorganisms on the 'resistant polysaccharides'. During the period 1970–75 at James Cook University the results were published of a series of investigations of other types of factors which might be envisaged as having potential to cause similar protection from digestion of some of the cell-wall polysaccharides. This review will survey the results of this series of investigations.

In the case of cellulose the molecule is unequivocally homogeneous. Each glucose unit is linked $\beta(1\rightarrow4)$ to its neighbour and there are no branches in the molecule. The most likely 'non-lignin' factor which might contribute to protection from digestion was therefore seen to be the supramolecular crystalline structure of the fibrils. In the commonly accepted microfibrillar structure for cellulose (ASPINALL, 1970; BIKALES and SEGAL, 1971) the crystalline or ordered regions (usually more than half of the weight of cellulose) are resistant to penetration and attack by small molecules such as water. It is certain therefore that they would resist penetration and attack by most hydrolytic enzymes. Obviously the ordered regions are penetrated by the C-factor component of the cellulase enzyme complex of cellulolytic micro-organisms, but conditions could be envisaged in the rumen which inhibit or restrict the functioning of the C-factor and hence limit the digestion of cellulose. Experiments were therefore devised to investigate the effect of cellulose crystallinity on digestibility.

In the case of hemicelluloses, the second major type of component of cell-walls,

* This is a report.

we have to deal with a mixture of different chemical types of polysaccharides. The first requirement therefore was to achieve an unequivocal measure of the actual rate and extent of digestion in the rumen of each of the several chemical types of hemicellulose in the intact cell-wall. Having confirmed that all types of hemicellulose were partially resistant to digestion, the next step was to consider possible 'non-lignin' mechanisms for this resistance. Since all of the hemicellulose molecules are branched, it seemed possible that the resistance might be at least partly associated with the formation of enzyme-resistant macrodextrins. Such molecules could be produced in two different ways, either by progressive removal of glycoses or oligosaccharides by exoenzymes from the non-reducing ends of hemicellulose molecules until the degradation reached a resistant branch point, or alternatively the macrodextrin could result from the presence in the hemicellulose of 'crowded' regions in which closely spaced branch points caused physical hindrence to access by hydrolase enzymes. Both possibilities have analogies in starch biochemistry, viz. the β-amylase limit dextrins (GREENWOOD and MILNE, 1968) and α-amylase macrodextrins (SCHRAMM, 1968) respectively.

Pectic substances are also major cell-wall constituents of some forages (ALAM and RICHARDS, 1971), but these have been shown to be rapidly and much more completely digested (DEKKER *et al.*, 1972).

METHODOLOGY IN DIGESTION STUDIES

In this type of detailed study, where for example we are concerned with relative rates of digestion of different components of the hemicellulose group, it is important that the digestion conditions are as realistic as possible. This implies working with forage cells *in vivo* in the rumen. The 'nylon bag' approach has therefore been used (PLAYNE *et al*, 1972) where the freeze-dried and chopped forage is enclosed in a cloth bag in the rumen. The bag is assumed fully permeable to the micro-organisms and the weight of material lost from the contents is assumed 'digested'. The different chemical types of hemicellulose are very difficult to fractionate unequivocally (BLAKE and RICHARDS, 1971) and the approach has been to determine the absolute glycose composition of the forage before and during digestion. The analysis involves complete acid hydrolysis of a sample, formation of suitable derivatives, and subsequent gas chromatographic analysis. In this way the rates and extents of digestion of the different chemical types of hemicellulose are assumed from the changes in different glycose components. This approach is straightforward in some cases, e.g. xylan content is probably a good general indication of total hemicellulose content since the great majority of the hemicellulose in grasses and legumes has been shown to be based on the β (1→4)-xylan backbone. In other cases, e.g. arabinan content, there are problems in detailed interpretation of results since the arabinose may be a component of both hemicellulose and pectic substances. In the latter case the pectic

substances may best be analysed in an approximate sense by selective extraction (e.g. by oxalate: PANG WAY and RICHARDS, 1971).

In order to compare the resistant hemicelluloses with the original, the hemicelluloses have been isolated from forage before and after digestion in the rumen and from undigested fibre isolated from faeces. The original and 'resistant' hemicelluloses have then been compared by compositional analysis, fractionation, and various physical methods.

THE EFFECT OF CRYSTALLINITY IN CELLULOSE

When pure cellulose (cotton seed hairs) is placed in the rumen, the rate of weight lost is very slow initially, but after about ten hours, more rapid digestion commences and proceeds to completion in about six days if the cotton is left in the rumen (BEVERIDGE and RICHARDS, 1975). The lag may be associated with a requirement for the cellulolytic bacteria to become attached to and grow viable colonies on the fibre before dissolution commences. During the progress of digestion the crystalline or ordered content of the undigested cotton has been followed by X-ray diffraction (BEVERIDGE and RICHARDS, 1975). The crystallinity decreases only slightly during the whole process and hence it is concluded that the amorphous and crystalline regions are attacked at similar rates. This conclusion is supported by the observation that the degree of polymerisation (d.p.) of the residual cellulose decreases only slowly during the process (from an initial d.p. 3760 to 2700 after 136 hours) since any preferential attack on the amorphous regions (as in acid hydrolysis (SHARPLES, 1971) or in attack by brown-rot fung (COWLING and BROWN, 1969) would cause a rapid fall in d.p. to about 200. The α-cellulose from spear grass, from rumendigested spear grass, and from an appropriate faeces fibre, were also isolated and shown to have similar d.p. and crystallinity (BEVERIDGE and RICHARDS, 1975). It must be concluded therefore that the crystallinity of the cellulose in the forage plays no significant part in its partial resistance to digestion in the rumen.

THE EFFECT OF MOLECULAR STRUCTURE IN HEMICELLULOSES

The most convincing experiments have perhaps been those in which hemicelluloses have been isolated from spear grass and partially digested spear grass (BEVERIDGE and RICHARDS, 1973). Comparisons have also been made of hemicelluloses from fodder containing predominantly spear grass and from the corresponding faeces fibre (BEVERIDGE and RICHARDS, 1973). In these cases the hemicelluloses were isolated by extraction with alkali and fractionated into B and C fractions (precipitable and nonprecipitable by ethanol respectively). The acetyl contents of the fibres were also determined, since acetyl groups are frequently found on hemicellulose molecules and would be lost during the alkaline extraction. The hemicellulose-B fractions were

Table 1. Component composition of the samples.

Component	Pure spear grass	Digested spear grass	Intake fibre	Faeces fibre
Lignin content of original sample[a] (corrected for ash and N)	15.1	20.5	18.0	25.5
Acetyl content of fibres[b]	1.5	1.6	1.9	2.1
α-Cellulose[a]	49.0	47.4	46.5	41.5
Hemicellulose B[a]	31.2	35.5	32.2	37.4
Hemicellulose C[a] (by difference)	19.8	16.0	21.3	21.0
Linear hemicellulose[c]	70.0	69.0	73.0	84.0
Branched hemicellulose[c]	30.0	31.0	27.0	16.0

[a] Expressed as percentage of holocellulose. [b] Expressed as percentage of washed starting-material.
[c] Expressed as percentage of combined weight of branched and linear hemicellulose.

further resolved by iodine complexing (GAILLARD, 1961) into linear and branched fractions. Results are shown in Table 1.

The digested samples showed higher percentage hemicellulose content and lower cellulose contents than the original grass because the cellulose digests rather more rapidly and completely than hemicellulose xylans (DEKKER *et al*, 1972), while the lignin is less digested than both.

The acetyl contents were very low and little changed during digestion. It was concluded therefore that they had no influence in digestion resistance. The ratio of linear to branched fractions in the hemicellulose of pure spear grass showed no significant change and it was therefore concluded that the resistance to digestion was not associated with formation of any analogue of starch β-limit dextrin, resistant to exo-enzyme attack. The change in proportion of branched hemicellulose in the faeces fibre is regarded as less reliable since some minor proportions of other fodder species may influence the results. The overall conclusions however, must be quite contrary to the earlier observation of BAILEY and co-workers (1965a, b), who found that branched hemicelluloses were digested much more slowly than linear components by cell-free extracts from rumen protozoa and bacteria. Subsequently (BEVERIDGE and RICHARDS, 1973b), the rates of digestion of the linear and branched hemicelluloses from spear grass were investigated using cell-free enzymes from the rumen micro-organisms. In this case no significant difference was found and it was concluded that in the earlier work (GAILLARD *et al*, 1965; BAILEY and GAILLARD, 1965b) the apparent more rapid digestion of the linear hemicelluloses was due to the rapid digestion of a contaminating glucan in this fraction and that there is in fact little difference in the true rate of digestion of linear and branched hemicelluloses in the rumen.

The compositions of the hemicellulose fractions are summarized in Table 2. The composition of the linear and branched hemicelluloses in the original spear grass shows the same trends as those reported by (GAILLARD, 1965) for a temperate grass.

Table 2. Glycose compositional analysis of samples.

Sample	Glc	Xyl	Ara	Gal	Uronic acid[a]	Xyl + Ara[b]	Ara/ Xyl
Spear Grass							
Water-washed fibre	65.2	28.0	5.8	1.0	–	33.8	17/83
Holocellulose	63.6	29.4	6.0	1.0	–	35.4	17/83
Hemicellulose B	5.0	73.4	14.0	2.7	4.9	87.4	16/84
Branched hemicellulose	4.3	66.7	22.0	tr	7.0	88.7	25/75
Linear hemicellulose	8.8	77.6	11.0	tr	2.6	88.6	12/88
α-Cellulose	92.3	5.6	2.1	–	–	7.7	27/73
Digested spear grass							
Digested fibre	60.6	32.4	6.0	1.0	–	38.4	15/85
Holocellulose	59.0	33.6	6.4	1.0	–	40.0	16/84
Hemicellulose B	4.2	75.4	14.4	2.5	3.5	89.8	16/84
Branched hemicellulose	1.8	71.0	19.2	1.9	6.1	90.2	21/79
Linear hemicellulose	3.1	83.8	10.3	tr	2.8	94.1	11/89
α-Cellulose	94.0	4.5	1.5	–	–	6.0	25/75
Intake fibre							
Total fibre	62.6	30.1	6.3	1.0	–	36.4	17/83
Holocellulose	64.2	28.8	6.0	1.0	–	34.8	17/83
Hemicellulose B	3.9	75.5	14.8	1.2	4.6	90.3	16/84
Branched hemicellulose	3.3	60.0	24.0	4.5	8.2	84.0	28/72
Linear hemicellulose	4.1	81.1	11.5	tr	3.3	92.6	12/88
α-Cellulose	92.9	5.4	1.7	–	–	7.1	24/76
Faeces fibre							
Total fibre	50.5	41.1	7.4	1.0	–	48.5	15/85
Holocellulose	48.6	43.3	7.1	1.0	–	50.4	14/86
Hemicellulose B	1.1	82.7	13.0	1.0	2.2	96.7	14/86
Branched hemicellulose	1.0	73.6	19.5	tr	5.9	93.1	21/79
Linear hemicellulose	1.1	85.7	11.0	tr	2.2	96.7	11/89
α-Cellulose	93.0	5.5	1.5	–	–	7.0	21/79

[a] By the carbazole technique. [b] Expresses a measure of hemicellulose content.
tr = trace – = not analyzed.

In particular, both uronic acid and arabinose contents of the branched hemicellulose are higher than the linear hemicellulose by a factor of 2–3. In the digested samples the branched fractions show a lower ratio of arabinose to xylose than the undigested samples, thus confirming earlier experiments (DEKKER and RICHARDS, 1973), which showed that the arabinose residues of the hemicellulose were digested more extensively than the xylose (e.g. 45% and 30%, respectively). The linear hemicellulose fractions however, showed no significant change in arabinose-xylose ratio as a result of digestion. The α-celluloses from the four samples contained 6–7% of non-glucose residues

which were quite resistant to digestion. The ratio of arabinose to xylose in this component was relatively high and approximated to that of the branched hemicellulose fractions.

Little significant difference therefore was found in the composition of the hemicelluloses originally present in forage and those which survived digestion. The fact that the resistant hemicelluloses from faeces fibre contained an increased proportion of 'linear' component may be associated with the previous observation that the arabinose component which is responsible for many of the branch points is more completely digested than xylose. If, for example, the resistance to digestion were associated with inhibition of exo-enzyme by branch points, we would anticipate more significant differences in composition between the original and resistant hemicelluloses. Such an effect would also have been expected to result in an increase in content of the predominant branching units (viz. arabinose and uronic acid), rather than the reverse.

Conclusion

The work summarized above has shown that at least for the particular case of digestion of spear grass in the bovine rumen, the resistance of cellulose to digestion is not related in any way to its crystalline structure. The resistance of hemicelluloses is not related to any molecular feature such as acetylation, molecular branching or chemical composition. The inevitable conclusion is that the resistance of the cell-wall polysaccharides to digestion is due to protection by the undigested portion of the lignin. Since a large proportion of the lignin dissolves during digestion in the rumen (Dekker *et al*, 1972) to give a soluble lignin-carbohydrate complex (Gaillard and Richards, 1975), it is possible that the resistant lignin is predominantly that which is not bonded to carbohydrate. If this is the case, then the protection of the 'resistant' polysaccharides is dependent on prevention, by lignified regions, of the attachment of the rumen micro-organisms to the regions containing the residual polysaccharides. In general the micro-organisms have to become physically attached to the plant cell wall before digestion can proceed, because very few extracellular enzymes are present (Beveridge and Richards, 1973) and the type of protection outlined above would be expected to be more predominant in some tissues than in others. The further study of this problem would be most appropriately carried out by microscopic studies of the digestion process and good progress is already being made in this direction (Akin and Amos, 1975).

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THE ESTIMATION OF DIGESTIBILITY FROM CHEMICAL COMPOSITION

PETER J. VAN SOEST

Cornell University, Ithaca, N.Y. 14853

SUMMARY

Research on the composition of forages has shown that the truly indigestible and unavailable fractions are quantitatively present in the plant cell wall. While various cell wall factors are associated with digestibility and voluntary intake, the interrelationships are complex and cannot be reliably predicted by any single analysis.

The conventional crude fiber system is seriously defective and is not historically correctly reported in the literature. The ultimate accounting of the digestibility of plant cell wall in terms of chemical structure and linkage remains undiscovered.

INTRODUCTION

The evaluation of animal foods for their available energy has always been based on methods that attempt to assay indigestible fractions. While digestion trials give an absolute measurement of availability, they are too lengthy and expensive and short-cut laboratory procedures have been sought and practiced. Historically, methods of evaluation have been based on plant fiber, on the assumption that it was somehow related to digestibility. In recent years this problem has been clarified with the demonstration that the plant cell wall, in fact, contains the truly indigestible matter in the diet.

This proof is perhaps the single most important advance in nutritional science since the discovery of the vitamins, for it has destroyed certain perennial theories about fiber and demonstrated that dietary fiber is a fundamental dietary characteristic and not a relative number that can be applied to all herbivore diets: ruminant, non-ruminant and human as well (SOUTHGATE, 1973; VAN SOEST and MCQUEEN, 1973).

The demonstration that dietary fiber recovers all truly indigestible matter and consequently that all non-cell wall matter has complete nutritional availability uninfluenced by the fiber itself has been obtained from two independent methods of study. The first through direct chemical fractionation of feed and feces for various carbohydrate fractions (GAILLARD, 1962; JARRIGE, 1965) and the second by statistical analysis of the digestible fractions of non cell wall and cell wall components (VAN SOEST, 1967). The results of such investigations are shown in table 1. The reason for the erroneous thought that lignified fiber might protect protein and non-structural

Table 1. True digestibility of forage fractions.

	Direct fractionation	Lucas analysis
Sugars (monosaccharides)	100[a]	100[b]
Fructosans	100[a]	–
Starch	98–100[c]	–
Pectin	95– 99[a]	
Protein	90– 95[d]	80–93[e]
Total non cell wall	–	98[e]
Hemicelluloses		50–79[e]
Arabans	60– 95[a]	–
Xylans	28– 72[a]	–
Aldobiuronic acid	64– 79[a]	–
Cellulose	40– 79[a]	43–80[e]
Lignin	1– 9[a]	0– 3[e]

[a] GAILLARD (1962).
[b] LUCAS et al (1961).
[c] At maintenance. Higher intakes result in lower digestibility (WALDO 1973).
[d] Pepsin solubility (GOERING et al 1972). Lower values indicate heat damage. Nitrogenous fractions resistant to digestion are recovered in acid detergent fiber and crude lignin (VAN SOEST, 1965b).
[e] VAN SOEST (1967) and COMBILLAS, GONZALES and PARRA (1971).

carbohydrate by encapsulation and cause their loss in feces is that dietary fiber supports a microbial fermentation in the rumen and the lower tract. About 30 percent of this microbial matter is indigestible and is lost in the feces and conventional methods have confused the identity of dietary and fecal substances.

The significance of these findings in the evaluation of forages is that the estimation of digestibility can be narrowed to two problems: (1) the methods for determining dietary fiber and (2) the study of factors that influence the availability of plant cell wall carbohydrates to anaerobic microbial digestion. The identification of plant cell wall with total dietary fiber also provides a criterion for the evaluation of fiber methods, in particular, crude fiber which still presents a problem because it embodies persistant archaic and erroneous concepts of dietary fiber. Since crude fiber is still the official method for food and feed analyses, an historical account of these misconceptions is relevant.

Many modern nutritional text books incorrectly attribute the crude fiber method to HENNEBERG and STOHMANN (1860) who plainly stated that it was adapted from EINHOF (1778–1808). HENNEBERG may be in error since a search through extant EINHOF papers fails to reveal a method based on sequential extraction with solvent, dilute acid and dilute alkali (TYLER, 1975). Instead, EINHOF (1806) published values obtained by a masceration and extraction with water which contrast with crude fiber values (table 2). The oldest extant value of fiber that compares with crude fiber is that of GORHAM (1820) who used alkali extraction alone.

Table 2. Some early fiber values compared with modern analyses (values are on dry matter basis).

Source	Barley	Corn	Oats	Potatoes (peeled)	Rye	Wheat	Meadow hay
	(%)	(%)	(%)	(%)	(%)	(%)	(%)
EINHOF (1806)	21.3	–	32.8	5.6	22	13.8	43[a]
GORHAM (1820)	–	3.3	–	–	–	–	–
HORSFORD (1846)	5.3	–	16.1	–	–	–	–
WOLFF (1856)[b]	4.9	–	12–16	–	2.4	1.6	25–34
MORRISON (1956)	6.0	2.4	13.5	2.2	2.7	2.9	26–34[c]
VAN SOEST (1973)[d]	21	12	31	4.7	–	14	40–55[e]

[a] Quoted from THAER (1809) assuming 12% moisture.
[b] WOLFF's values are from summaries of earlier literature
[c] Mixed hay.
[d] Neutral-detergent fiber.
[e] Mixed clovers and grasses.

The early chemists, EINHOF included, thought that fiber was indigestible and actually calculated nutritive value in terms of hay equivalents (THAER, 1809; WOLFF, 1856). The possible origin of this idea may have been from the experimental retting of flax using acid and alkali in the eighteenth century, (WILSON 1853). All this preceded the earliest digestion trials by HAUBNER (1855) who demonstrated the digestibility of fiber, the vital information that led to the abandonment of hay equivalents (HENNEBERG, 1859).

The problem thus arises as to why the crude fiber procedure was adopted over that of EINHOF. Some idea is given by HORSFORD (1846) who generally used alkali to prepare fiber and acid only as judged necessary. Three criteria of fiber purity may be summarized: residual ash and nitrogen should be minimal and the carbon, hydrogen and oxygen composition should be that of pure carbohydrate.

Here lies the central problem of fiber analysis that is with us today. Should the method be characterized in terms of distinct, pure chemical entities, *viz.* cellulose, lignin; or rather recover sundry indigestible matter in a heterogenous residue? The latter approach is represented by the trend to define dietary fiber as plant cell wall, a residue that recovers most of the truly indigestible matter. The use of individual cell wall components such as lignin to estimate digestibility through regression equations represents a persistence of the chemical entity concept.

The lack of recovery of indigestible matter in crude fiber has been the source of much criticism of the method. In about 30 percent of the tabular values of feedstuffs, crude fiber is as digestible or more digestible than the nitrogen-free extract, though the main purpose of their division was to separate the unavailable from the available nutrients. The principal fractions that are not recovered in crude fiber are hemicelluloses and lignin (table 3). The unrecovered amounts of lignin and cellulose

Table 3. Percentages of lignin, pentosans and cellulose dissolved in the crude fiber determination.

Sample	Lignin	Pentosans	Cellulose
	%	%	%
Legumes			
Alfalfa	50	86	39
Red clover	19	82	34
Sweet clover	8	–	39
Vetch	62	–	27
Bean straw	33	–	16
Peanut hulls	9	21	12
Grasses			
Bermuda grass	95	–	17
Cheat grass	89	–	22
Rye grass	94	–	26
Timothy	72	68	5
Maize plant	90	–	40
Wheat plant	86	–	29
Oat straw	75	79	24
Rye straw	77	64	7
Wheat bran	53	89	22
Other			
Bovine feces	76	84	–
Kale	45	–	28
Silage	84	80	–
Sugar beet pulp	78	–	32
Swedes (peeled)	57	64	7
Spruce wood	10	43	30
Yarrow	45	–	15

Summarized from ARMSTRONG et al. (1950) KIM et al. (1967), NORDFELT et al. (1949). PALOHEIMO and MÄKELA (1959) NORMAN (1935) and STALLCUP et al. (1964),

are quite variable and depend on the type of plant, more lignin being dissolved from grasses than from legumes. Generally hemicelluloses are dissolved to about 80 percent. The proportions of lignin, cellulose and hemicelluloses vary within plant cell walls, leading to wide variation in the yield of crude fiber relative to plant cell wall (figure 1).

This lack of relationship indicates the hazard of the continual use of crude fiber as a measure of dietary fiber and emphasizes the errors if it is presumed that crude fiber is at least correlated with dietary fiber. This hope may hold true to a limited extent within plant families such as the Graminae or Leguminosae but disappears into chaos as one crosses the agronomic lines. The regularity that exists within a group depends upon characteristic proportions of lignin, cellulose and hemicelluloses (VAN SOEST 1973). Since cellulose tends to be better recovered than lignin or hemicelluloses, the

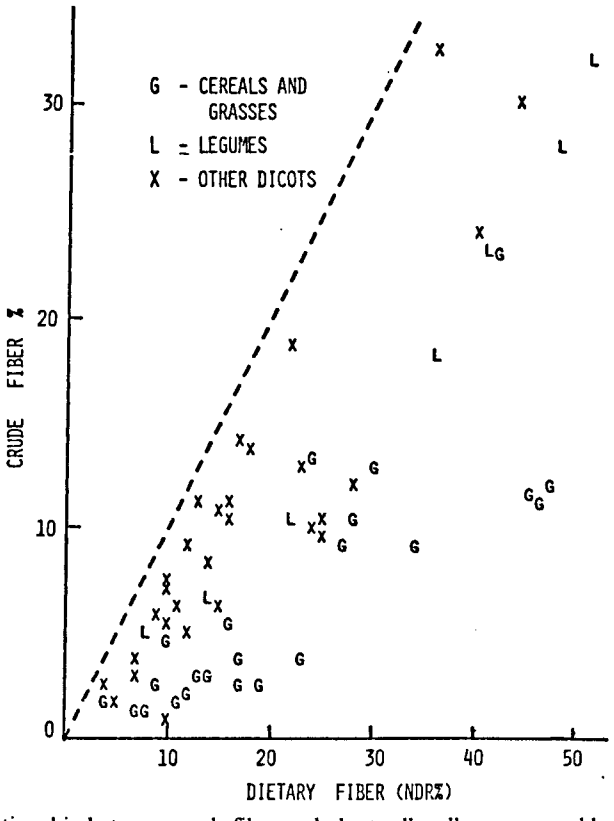

Figure 1. The relationship between crude fiber and plant cell wall as measured by neutral-detergent residue. (Unpublished data of Van Soest and Robertson).

relationship between crude fiber and dietary fiber is dependent to a considerable extent upon the proportion of cellulose in the plant cell wall.

Digestibility of the cell wall

Because of the complete availability of the non cell wall components, the problem of estimating forage digestibility is essentially that of estimating cell wall digestibility. Regression methods depend upon factors that may estimate digestible cell wall. If fiber is used its success depends upon the degree of correlation between its content and its own digestible quantity (Lucas *et al*, 1961; van Soest 1973). Most fiber fractions fail this test with the exception of lignin.

The source of variability in fiber digestibility has long been regarded as being mainly plant maturity, but other sources of variation exist as well, such as environmental and genetic differences among plants. Estimation of digestibility with fiber

fractions works better where the variation in maturity is emphasized and the environmental and genetic ones are small. As the variation in environmental and genetic factors increases the associations between lignin and cellulose decline, and even the use of lignin which has been the best of the estimators of digestibility causes considerable error in the prediction. The error with the use of lignin is particulary increased through the interaction of legumes and grasses. Grasses tend to have a higher cell wall content and lower lignin content than legumes at the same digestibility (VAN Soest 1973). These differences are largely responsible for the association or lack of association between digestibility and voluntary intake.

While the plant cell wall quantitatively contains the truly indigestible portion of the diet, ironically it is poorly related to digestibility (table 4) and is better related to voluntary intake. This arises because of the association between structural volume and cell wall content (VAN SOEST, 1975) and the large variation in lignification and other factors which limit digestion of the plant cell wall. The result of this variability

Table 4. Correlations between composition of forage, voluntary intake and digestibility.

Component	Number of forages	Voluntary intake	Dry matter digestibility
Cell wall	14[a]	−.66	
	77[b]	−.56	−.32
	187[c]	−.77	−.45
	83[d]	−.65	−.65
Acid detergent fiber	14[a]	−.13	
	77[b]	−.31	−.74
	187[c]	−.64	−.75
	83[d]	−.53	−.74
Lignin	14[a]	−.09	
	77[b]	+.21	−.64
	187[c]	−.08	−.61
	83[d]	−.13	−.52
Cellulose	14[a]	−.14	
	187[c]	−.75	−.56
	83[d]	−.59	
Dry matter digestibility (in vivo)	77[b]	+0.8	
	187[c]	+.44	
	83[d]	+.66	
Dry matter digestibility (in vitro)	187[c]	+.47	+.80

[a] BRAHMAKSHATRIYA, (1971).
[b] JOHNSON and DEHORITY (1968).
[c] MERTENS (1973).
[d] VAN SOEST (1965a).

in cell wall quality is that compositional factors that are usually associated with intake are less well associated with digestibility and *vice versa*. Thus, for example, cell wall and cellulose are better associated with intake while lignin, acid detergent fiber and *in vitro* digestion are more related to digestibility (table 4). Experience has always been that *in vitro* digestibility has the best relationship to digestibility, better than any chemical assay. This arises because the rumen organisms respond to undetermined factors present in the forage cell walls.

The relationship between *in vivo* and *in vitro* digestibility is probably affected by the manner in which the digestion trials are conducted. Digestion trials conducted in conjunction with *ad libitum* intake measurements involve a feed refusal and a variation in intake level which influences the rate of passage and digestibility. Generally, increased rate of passage is associated with a drop in dry matter digestibility that is almost quantitively expressed in the plant cell wall carbohydrate fraction (VAN SOEST, 1975). The correlation value of .80 between *in vivo ad libitum* digestibility and *in vitro* digestibility is generally lower than those reported in the literature based on restricted intakes.

The problem of estimating digestibility from chemical composition remains because there is no chemical method that will fractionate the carbohydrates of the plant cell wall into those available and unavailable. It is only by utilizing regression models, or *in vitro* systems using rumen organisms or cellulase enzymes (MCQUEEN and VAN SOEST, 1975) that estimates can be made. Regression models utilizing summative calculations (VAN SOEST, 1967) have disclosed a variety of factors that effect digestibility. These include lignin, cutin, silica and polyphenols (VAN SOEST and JONES, 1968; GAILLARD and RICHARDS, 1975; VAN SOEST, 1975) and also the uronic acid components of the hemicelluloses (GAILLARD, 1966). The difficulty presented is that while such models may estimate digestibility very satisfactorily provided the right measurements are made, the number of determinations and the laboratory work is large and expensive.

In addition, there are structural features of the plant cell wall, as yet undiscovered, which tend to leave the practical evaluation to the use of *in vitro* rumen fermentations. Cellulases and hemicellulases are more variable (MCQUEEN and VAN SOEST, 1975) than are rumen organisms and a development of a better understanding of these enzymes as well as classification of the factors and linkages attacked by them will be an important area of research in the future.

The presence of hemicellulosic linkages to lignin and the presence of lignins soluble through enzymatic action upon grass cell walls (HARTLEY, 1972, GAILLARD and RICHARDS, 1975) offer new insight into these problems as well as the potential development of laboratory methods that could have some practicality.

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