STRUCTURAL STUDIES

IN THE

HEMICELLULOSE GROUP

by

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### INTRODUCTION

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It is impossible to frame a definition of the term hemicellulose that has strict analytical significance without making it cumbersome. Many definitions have been advanced some having a chemical, some a physical, others a cytological basis, but there is none that would gain the unqualified acceptance of all workers in the field. One widely accepted definition, which is essentially the same as that originally suggested by Schulze (1), may be stated as follows: The hemicelluloses are land-plant polysaccharides which are not readily extractable from plant tissues, either before or after delignification, by treatment with cold water but which may be extracted with dilute alkali. They are, furthermore, much more readily hydrolysed by dilute mineral acids than is cotton cellulose. Such a definition has the merit that it does not presuppose a knowledge of the chemical nature of the molecular species to be found in the plant, nor does it anticipate a knowledge of the place of origin, or the function, of the hemicelluloses in plant tissue. It suffers from the grave drawback that a variation in the extraction technique leads to a variation in the amount of material that may be termed hemicellulosic. Under the conditions normally employed, namely treatment of the holocellulose (p. 63) with 4% sodium hydroxide, a major proportion, but by no means all, of the alkali-soluble material passes into solution. This prompted
Schmidt (2) to divide the hemicelluloses into two types: the 'easily soluble' and the 'difficultly soluble'.

Norman (3) divided the hemicelluloses into two classes: the cellulosans and the polyuronides. The former he believed were pentosans, hexosans or hexopentosans and were associated with the plant cell-wall. The latter, in addition to the above, also contained uronic, or partly methylated uronic, anhydride residues. Such a division of the hemicelluloses is necessarily artificial and liable to alteration as a knowledge of the structure of the molecules, and their relationship to other molecular species in the tissues, is extended.

An indication of the complexity which is to be expected in any investigation of the hemicelluloses, and an explanation of the difficulties encountered in their fractional extraction, may be gained from a consideration of the structure of plant tissues (4-6).

Newly formed higher plant cells consist of a protoplast surrounded by a very thin solid envelope, the primary wall, which has been shown to consist of cellulose, pectic substances, and possibly varying amounts of hemicelluloses. Growth of the cell involves the expansion of this primary wall and is followed by its fission to yield daughter cells. Between the primary walls of these cells, that is in the middle lamella, there is deposited, or elaborated, inter-
cellular material one of the primary functions of which is to act as a matrix binding the cells together. Meristimatic cells, that is cells still capable of growth, have only a primary wall surrounding the protoplast. When the cell has reached its mature shape and size the formation of an internal, secondary, wall takes place.

The middle lamella has been shown to consist principally of lignin with smaller amounts of polyuronides; in the case of Douglas fir, A.J. Bailey (7), following microdissection, found that they were present to the extent of 71% and 14% respectively.

In maturity the primary wall is difficult to investigate apart from the secondary wall.

The central and outer secondary walls (8) are primarily made up of true cellulose the amount of which falls off from inner to outer regions of the walls while the amount
of pentosan increases. I.W. Bailey believes that polyuronides containing xylose are present therein. The inner secondary wall is principally proteinaceous and a derivative of the protoplast.

Hemicelluloses containing D-xylose, D-mannose and uronic anhydride residues commonly occur in all plant tissues. L-arabinose, D-galactose and L-rhamnose are found to a smaller extent. In annual plants and in hard woods xylan is the principal hemicellulose but in soft woods it appears to be partly replaced by mannan.

In 1846 Poumarède (9) noted that a substance, wood gum, could be extracted with alkali from wood. Chemical investigation of the hemicelluloses started with the work of Wheeler and Tollens (10) on those from beech wood. They found the acid hydrolysate to contain xylose. The spate of work (11) which followed in the next few years yielded useful information on the hydrolysis products of the hemicelluloses but shed little light on the structure of the individual molecules. The fundamental difficulty lay, and indeed lies, in the trouble which is encountered in any attempt to prepare a pure hemicellulose. The material obtained by simple alkaline extraction of a holocellulose commonly contains a mixture of different molecular species. Due to their frequently similar chemical and physical properties the problem of obtaining a separation is often one of great difficulty.
On the other hand different physical properties, such as solubility, may be found in one hemicellulose species due to its wide molecular weight distribution. A final complication, and one that even yet cannot be considered as being in any way overcome, is that of the separation of molecules that differ only slightly in their fundamental building units. Failure to obtain a homogeneous starting material naturally greatly reduces the value of any structural work carried out. Re-examination of much earlier work has indicated that in a great many cases it was carried out on heterogeneous material and some of the conclusions drawn must therefore be considered either spurious or at least suspect.

It would be unsatisfactory to consider the hemicelluloses apart from other molecular species with which they may be bonded. Much evidence has accumulated which suggests the possibility of bonding of the hemicelluloses to lignin and, separately, to cellulose.

**Relationship of the Hemicelluloses to other Molecular Species in Plant Tissues.**

It has frequently been observed that it is very difficult, and in many cases apparently impossible, to free α-cellulose from pentose residues. Various hypothesis to account for this difficulty have been propounded but the evidence is either conflicting or uncertain.
Astbury, Preston, and Norman (12) thought that the pentose-α-cellulose relationship might be analogous to that of a solid solution. Hawley, Norman (13), and Schmidt (14) suggested that there might be a definite chemical linkage between the cellulose and the xylan or that there might be a glucoxylan. Irvine, and Hirst (15), however, obtained no evidence of the existence of a glucose-xylose disaccharide in the hydrolysate of esparto grass holocellulose. Sarkar, Mazumdar and Pal (16,17) working on jute α-cellulose could detect no evidence of a chemical bond of any nature, although three years later Das, Mitra and Wareham (18) by graduated acid-hydrolysis of jute α-cellulose obtained an oligosaccharide which on hydrolysis gave glucose, xylose, and arabinose. It is of interest to note that the oligosaccharide travelled on paper chromatograms at a rate which indicated that it was either a trisaccharide or a tetrasaccharide and was probably the former. It therefore appeared that there was some molecular species in which glucose, arabinose and xylose residues were present in close proximity. Das et al. (18,19) claimed that this conclusively proved the existence of a cellulose-pentosan link or the existence of a cellulosan. They do not appear to have considered the possibility that there may have been two oligosaccharides travelling at the same rate on their chromatograms one containing only glucose residues, the other only pentose residues. The work of Adams and
Bishop (20) agrees with that of Das. They found that if the glucose residues in oat, wheat, jute and barley c-celluloses were removed by digesting the c-celluloses with a glucose oxidase then arabinose and xylose could be detected in the residual matter.

Evidence has also been advanced in favour of the theory that the hemicelluloses are bound chemically to lignin and that, in particular, there is a xylan-lignin bond. It has been noted many times that the ease of dissolution of the hemicelluloses increases greatly when the plant tissues have been delignified. This may indicate the existence of a chemical bond between the hemicellulose and lignin or may be purely a mechanical effect due to the lignin blocking the access of the solvent to the tissue. It was noted by Jayme (21) and Wise (22) that as long as the percentage of lignin did not fall below 3% during chlorite delignification the hemicelluloses remained practically insoluble in the aqueous wash liquors. Further reduction in the amount of lignin present led to a considerable loss of hemicellulosic material by solution. Other work (23) lends support to the theory of the existence of such a bond. Hagglund (25) found that, in the process of preparing sulphite pulp, the lignin and hemicelluloses which at first were insoluble, at a later stage simultaneously passed, in part, into solution. Floetz (24) noted the
readiness with which an enzyme preparation attacked a cupriethylenediamine extract and residue of lindenwood, but did not attack the native wood. Kawamura (26) claims to have proved that lignin is chemically bound to xylobiose - a view that gained support from the work of Traynard and Ayroud (27), who, working with highly purefied lignins have discovered that they contain chemically bound xylose, arabinose and galactose.

There is therefore much evidence in favour of such a bond but, at the moment, it would be rash to state that it definitely exists. The fact that lignin is found to contain pentose units does not necessarily mean that these units were at one time part of pentosans or polyuronides.

General Methods.

Delignification.

The presence of lignin, as has already been mentioned, increases the difficulty of extracting the hemicelluloses with alkali from plant tissues. It is, also, a nuisance since it is to an extent soluble in alkali and, unless removed prior to the alkaline extraction of the hemicelluloses, poses a problem during the subsequent purification.
An early delignification method, devised by Norris and Preece (28), was to treat the lignified tissues with a boiling solution of ethanolic sodium hydroxide: a method which led to some degradation of uronic-acid-containing polysaccharides (29). Partial delignification has been obtained by certain workers by treating the 'dewaxed' plantstuffs with alkali of a dilution insufficient to dissolve the hemicelluloses to more than a slight extent (30). Schmidt (31) removed lignin by treatment with chlorine dioxide in pyridine and water. Ritter and Kurth (32) first chlorinated, and then extracted the lignin product with ethanol and then with an ethanolic solution of ethanolamine.

The method which in recent years has, in one or other of its modifications (33), been most widely employed is that originally due to Jayme (34). He delignified sawdusts by treating them with aqueous solutions of acetic acid and sodium chlorite. This method has since been very widely applied to the delignification of woods and annual plants.

Harwood (35) has criticised the use of aqueous acetic acid-sodium chlorite solutions for delignification purposes. In a study of the action of such solutions on wheat straw he found that, after nine hours treatment at 75°, 58% of the xylose, 55% of the arabinose, and 17% of the glucose residues in the original straw had passed into solution in
the chlorite liquors. It is uncertain to what extent this dissolution was dependent on degradation of the polysaccharide material in the holocellulose, and to what extent it was due to an increase in ease of access of the solvent to the hemicelluloses—coupled, possibly with the rupture of a lignin-hemicellulose bond. The fact that polysaccharides and not free sugars were detected in the chlorite liquors suggests that the principal action may be to increase the solubility rather than to cause degradation. The conditions employed by Harwood were very drastic, at any rate for the delignification of a straw. It is almost certain that within the first hour the lignin would fall below the critical value of 3% and loss of hemicellulose would therefore be expected (p. 8).

Jayme and Mo (36), and Staudinger and Jurisch (37) believed that chlorine dioxide had little destructive action on wood pulps. Jeanes and Isbell (33) determined that the principal danger was that reducing groups might be oxidised. Such oxidation they noted was slow in neutral solution but rapid in acid solution. Whether or not the acid chlorite causes degradation is uncertain, but in spite of this uncertainty nearly all workers in recent years have chosen to use the method.
Extraction, Purification, and Fractionation of the Hemicelluloses.

The classical method of Pouinardé and Figuier (9) for the extraction of the hemicelluloses with alkali is still, in one or other of its modifications, the one normally employed. Aqueous sodium and potassium hydroxide solutions of strengths up to 24% (39) have been used. Most workers (48,82,88,90,94,95,97) have chosen to extract with 4% alkali at room temperature, sometimes air being excluded. The danger of alkaline oxidation of simple sugars was investigated by Frey, Waldmann and Krzandalsky (40). They, on the basis of their investigation of the effect of alkali on beechwood hemicelluloses under different conditions, state that the principal factor influencing degradation, as determined by measurement of the degree of polymerisation by physical methods, is temperature. They conclude that the presence of oxygen, the reaction time, and the degree of alkalinity appear to have little effect. The possibility must be borne in mind that the hemicelluloses investigated by these workers may have been degraded during the process of extraction. Gehman, Kreider, and Evans (41) and other workers (42) have noted that certain reducing oligosaccharides undergo alkaline degradation from the reducing end of the molecule. Kenner (43) has recently investigated the alkaline degradation of simple sugars in the absence of oxygen. He has noted, inter alia, that treatment of lactose at 25°C
with saturated lime water causes 1.9% decomposition after half an hour and this increases to 85.8% after 216 hours.

Other extractants which have been used include: water, cupriethylenediamine and aqueous solutions of sodium and potassium carbonates. Hess, and Gundermann (44) noted that liquid ammonia swells cellulosic fibres and would therefore be expected to render cellulosic material more accessible to solvents. Bishop, and Adams (45) found that if wheat straw were allowed to swell in liquid ammonia at its boiling-point (-33°C) then the water soluble portion increased from 3% to 20.3%. Björkqvist and Jürgensen (46) used this method to extract hemicelluloses from birch and spruce woods.

Many factors affect the readiness with which the hemicelluloses may be extracted. It is found that it is generally more difficult to extract them from old, or non-freeze-dried materials, than it is to extract them from fresh material, or material that was freeze-dried when fresh. Pretreatment of plantstuffs is often considered desirable as it enhances the ease with which the hemicelluloses may be extracted, it also minimises contamination of the hemicellulosic extract. The removal of one of the chief contaminants, lignin, has already been considered. Wax-like materials, fats and resins are removable by treating the plantstuffs with organic solvents such as methanol (47) and then benzene or with an azeotrope of
ethanol and benzene. By treatment with hot water, water-soluble carbohydrates and mineral matter may be removed; this may also result in a considerable loss of hemicellulosic material. Pectic materials are normally extracted by treating the 'dewaxed' tissues with 0.5% aqueous ammonium oxalate (49).

It is in many cases extremely difficult to obtain a homogeneous starting material and it is probable that much work has been carried out, especially prior to the introduction of chromatographic techniques, on heterogeneous materials. Separation difficulties arise not only from the presence of different kinds of chemically and physically similar molecules, but also from the occurrence of each molecular species in different degrees of polymerisation. It is improbable that any simple method based on solution or precipitation, which does not involve laborious and time-consuming fractionation techniques, can be expected to yield absolute separations.

Extraction by alkali of gradually increasing strength has been used (50,51), but by this means no perfect separation of alkali-soluble molecular species has been achieved. O'Dwyer (52) made the alkaline extract slightly acid and separated off the precipitate formed (hemicellulose A), then she neutralised the solution and added an excess of alcohol which resulted in the formation of a further precipitate (hemicellulose B). These fractions have been found to be
different in some cases and in others to be apparently the same.

Salkowski introduced the use of Fehling's solution (53) for the purification of xylans. He believed, at first, that the xylan formed a copper complex while the arabans present did not do so (54). This view he later modified (55) by suggesting that the arabans did form complexes but that they were preferentially dissolved by an excess of Fehling's solution. Modifications involving the use of glycerine and copper sulphate have been used (56) and both methods, especially the former, have been widely employed in efforts to reduce the arabinose/xylose ratio in hemicellulosic extracts.

Various workers (57-59) have chosen to acetylate the hemicelluloses and to fractionate the product from chloroform solution, into polysaccharides of decreasing molecular weights, by the addition of petroleum ether. Harwood (58) fractionally precipitated the hemicelluloses from cupriethylenediamine solution by the addition of acid and alcohol.

Most workers fractionate their 'purified' hemicellulose after it has been methylated. The primary reason for this fractionation is to obtain a fully methylated product, but it is probable that some fractionation of different molecular species may occur at the same time if the material was heterogeneous.
Carson, and Dayton Maclay (57) have prepared the acetate, propionate and butyrate of a lima bean hemi-cellulose. They found that fractionation of the lima bean esters from organic solvents failed to result in an appreciable lowering of the anhydro-glucose content and they concluded that it was probably chemically combined with the xylan.

Nomenclature.

Throughout the following pages the term 'xylan' is used to denote molecular species which consist principally of xylose residues and 'araboxylan' to describe those molecular species which have, in addition to xylose residues, a large proportion of arabinose residues.

Early Structural Work on Xylans (60,61).

Koch (62) and Wheeler and Tollens (65) showed that xylan on mild acid hydrolysis yielded a sugar identified as xylose. Endeavours to determine the empirical formula of xylan were frustrated by the difficulty experienced in freeing it from moisture of which it commonly contains from 8-11% under normal atmospheric conditions.
Johnson (11), in 1896, determined the formula to be \((C_5H_2O_4)_n\) and this was confirmed some thirty years later by Hampton, Haworth, and Hirst (63) and by Link (64).

Before it was realised that xylan was a polymer Wheeler and Tollens (169) advanced two inner anhydride formulae for it.

![Chemical Structure](image)

In 1895 Bader (66) prepared diacetyl and dinitro derivatives of xylan and from this concluded that the original molecule must have contained two free hydroxyl groups, a view which was substantiated when Heuser (67) prepared the dimethyl derivative in 1922. The following year Komatsu, Inoue and Nakai (68) hydrolysed methylated xylan and identified the resultant product as dimethyl-xylose by oxidising it to dimethoxy-glutaric acid. They suggested the following formula for xylan:

![Chemical Structure](image)

The experimental work which led to this formulation has been criticised.
Hampton, Haworth, and Hirst (63) hydrolysed methylated esparto xylan with methanolic hydrogen chloride and obtained a methyl di-α-methyl-D-xylopyranoside which, on further methylation followed by hydrolysis, yielded the known (69) 2:3:4-tri-α-methyl-D-xylose. It therefore followed that in the dimethyl xylose two of the positions 2, 3 and 4 were methylated. Since no osazone could be prepared from it, it was concluded that C(2) carried a methoxyl group. The dimethyl xylose (I) was oxidised with bromine and the lactone (II) prepared. Polarimetric study of the rate of hydrolysis of the lactone suggested that it was most probably a γ-lactone and that position C(4) of the dimethyl xylose was free.

Methylation of the lactone yielded the known (70) 2:3:5-tri-α-methyl-D-xylofuranolactone. It was obvious from the above that the methyl groups were on the 2 and the 3 position in the dimethyl xylose and that the xylose residues in xylan must be linked either 1-4 or 1-5. The latter linkage was that originally proposed by Komatsu et al. (68). Comparison of the high laevorotation of xylan with the rotations of the anomeric methyl-D-xylosides
indicated that the xylose residues were probably linked by β glycosidic bonds. Hampton and co-workers, in view of the comparative stability of the polysaccharide, favoured a pyranoid structure for xylan:

\[ \text{\includegraphics{structure1.png}} \]

Confirmation of the above structure was supplied by Haworth and Percival (71) in 1931, that is, two years later. Methylated esparto xylan was acetylated for ten minutes at 0°. One of the xylan fragments obtained on deacetylation was shown, by work to be outlined, to be a partly methylated xylobiose (I). Oxidation of this product (I) with bromine yielded a derivative of xylobionic acid (II) and from this the ester of hexa-O-methyl-D-xylobionic acid (III) was prepared by methylation and esterification.

\[ \text{\includegraphics{structure2.png}} \]
The ester on hydrolysis yielded the known 2:3:4-tri-O-methyl-D-xylose (IV) and 2:3:5-tri-O-methyl-D-xylonic acid (V). The latter had been characterised by its conversion to the lactone (VI) and thence to L-dimethoxy-succinic acid (VII).

The above work proved that C(5) took part in ring formation while C(4) was linked glycosidically to another xylose residue. More recently Jayme and Sátre (72)

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have proved that the basic structure is that suggested by Hampton and co-workers (p.19). Periodate ions will attack molecules which have two contiguous carbon atoms on which there are hydroxyl groups. Jayme isolated glyceraldehyde and glyoxal (III) in equimolecular amounts from the hydrolysate of periodate-oxidised xylan (II).

By 1931 the basic structure of xylan had been established. Succeeding years added complexity. Haworth, Hirst, and Oliver (73) re-examining the hydrolysate of methylated esparto xylan found it to contain 2:3:5-tri-\(\beta\)-methyl-L-arabinose. Mono-\(\beta\)-methyl-D-xylose was also found; there was considerable uncertainty about the significance of the latter. Was it produced by under-methylation of the xylan or was it structurally significant? So far no indication of the presence of a reducing group had been
found and on the basis of the information available several structures could be advanced (I-IV). They endeavoured to explain certain, or all, of those features noted by 1934:

1) the presence of monomethyl-xylose.
2) the absence of a reducing group.
3) the presence of trimethyl-arabinose.
4) the absence of trimethyl-xylose.
5) the fact that in esparto araboxylan the ratio of arabinose to xylose is 1:18-20.

Afl → ?Xpl $\& (4Xpl $\& 4Xpl $\&)_g$ -OH

Afl → ?Xpl $\& (4Xpl $\& 4Xpl $\&)_g$ -OH

Afl ....... → 4Xpl → 4Xpl → 

Xpl → [ (4Xpl) a $\& 4Xpl $\& (4Xpl) b ]_n -4Xpl -OH

Af

Af = arabofuranose residue.
Xp = xylopyranose residue.
Xpl-OH = xylopyranose reducing group.

a + b = 18

? = uncertain position of linkage.
Bywater, Haworth, Hirst and Feat (74) found that it was possible to remove the arabofuranose residues from esparto arabinoxylan by very mild acid hydrolysis (0.2% aqueous oxalic acid at 100°C). Methylation and hydrolysis of the resultant xylan yielded, inter alia, 7% of trimethylxylose and the authors concluded that it arose from the xylose residue which had, prior to the hydrolysis, been adjacent to the terminal xylose. This ruled out formulae (III) and (IV). The approximate equivalence of the amounts of end group and of monomethyl-xylose suggested that formula (II) best fitted the observations. They proved that 2-O-methyl-D-xylose was present in the methylated arabinoxylan hydrolysate but were uncertain of the nature of the linkage between chains A and B.

O'Dwyer obtained a xylan from heartwood which, she concluded, contained methylhexuronic acid residues (75). Glucuronic acid has been discovered in many xylans; details are given in the next section.

Much other work has been carried out on xylans; particularly notable work has been performed by O'Dwyer (76) and by Anderson (77) but their work, however, has been adequately reviewed elsewhere (78).
Recent Work on Xylans and Araboylans (60,78,79).

With the introduction of techniques based on chromatography, and more recently on ionophoresis, a powerful tool has been placed in the hands of the polysaccharide worker and within recent years much structural work utilising these methods has been carried out.

Wheat.

The xylans and araboxylns of wheat have been rigorously investigated and the result of this work is interesting from two points of view. Firstly, it permits comparison to be made of the structures of the pentosans and polyuronides from different parts of the plant, and has enabled Perlin (80) to advance a new theory regarding the transportation of the pentosans in the plant. Secondly, it emphasises the variability in structure of pentosans obtained from straw by techniques that differ only slightly. This variability may be due to lack of homogeneity in the pentosan investigated or may be due to degradation having taken place prior to structural determination. Other possibilities are that straws of different ages or varieties, or straws that have been grown under different conditions may contain structurally different hemicelluloses. There is, however, much evidence in favour of the theory that in the straw there is more than one hemicellulose containing xylose residues.
It is proposed to deal in some detail with the hemicelluloses from wheat as they are illustrative of the complexity of structural determinations and suggest that great caution is necessary in the interpretation of results.

Wheat straw.

Weihe and Phillips (81) delignified wheat straw by treating it with chlorine and extracting the chlorolignin product. The residual straw was then treated with alkali, and the hemicelluloses which were extracted thereby were precipitated from alkaline solution by the addition of ethanol. They obtained a xylan which on hydrolysis yielded an uronic acid, L-arabinose, and D-xylose in the ratio of 1:0.9:23.

Aspinall, and Mahomed (82), investigating the hemicelluloses obtained from an acid-chlorite delignified wheat straw, found them to contain arabinose, xylose, and glucuronic acid residues. The successive formation and decomposition of copper complexes failed to yield a xylan free from arabinose residues. Extraction of the hemicelluloses thus obtained with aqueous ethanol (70%) yielded a xylan which gave only a trace of arabinose on acid hydrolysis. By isothermal distillation of a solution of the methylated xylan they determined the degree of polymerisation of the methylated xylan to be 47-53, and from methylation studies they concluded that the
molecule contained one non-reducing xylose residue per 40-45 residues. These two facts in conjunction indicated that the xylan comprised an unbranched chain of xylose residues as shown in the following formula:

\[ \text{Xpl} \rightarrow (4\text{Xpl}) \rightarrow 4\text{Xpl} \rightarrow (4\text{Xpl}) \rightarrow 4\text{Xp} \]

\[ \begin{array}{c}
\text{GpA} \\
a+b = 40 \\
\text{GpA} = \text{glucuronic acid residue.} \\
\text{Xp} = \text{xylopyranose residue.}
\end{array} \]

Simultaneously, and independently, Adams (83,84,85) had been working on a xylan also from wheat straw. He concluded that it consisted of a chain of 32 xylose residues, linked \( \beta 1 \rightarrow 4 \), to which there were attached, as side-groups on the 3 position, five arabinose and three uronic acid residues.

Roudier (86) concluded that wheat straw xylan consisted of a chain of 41 xylose residues with one arabinose and one uronic acid residue as side-groups. The linkages were the same as found by Adams. Roudier determined that \( 4/5 \text{ths.} \) of the uronic anhydride was present as the mono-methylether.

Mahomed, Adams, and Roudier have reported that the uronic acid residues are attached to the \( C(3) \) position of xylose residues in the main xylan chain. All these workers relied on chromatographic identification of the mono-O-methyl-
D-xyloses which they obtained from acid hydrolysates of reduced, methylated aldobiuronic acids. Since 2 and 3 methyl xyloses have $R_G$ values which are very nearly the same in the chromatographic solvents used by the above workers, it seems possible that, on paper chromatograms, the spot produced by one methyl xylose on spraying the paper with a suitable reagent may have masked the spot produced by another methyl xylose. Bishop (87) in a subsequent paper has confirmed that in the straw xylan investigated by Adams the uronic acid was attached to a $C(3)$ position on the main xylan chain. Meek (88), however, has obtained evidence which suggests that the uronic acid residue may be attached to $C(2)$.

The most notable difference in the above structure is that Mahomed, unlike Adams or Roudier, found a xylan free from arabinose residues. Meek has recently separated an araboxylan from the same straw as Mahomed and has concluded that it definitely contains arabinose residues glycosidically attached to $C(3)$. A possibility that must be considered is that the arabinose found in 'xylan' hydrolysates may have been derived from an associated araban and not from an araboxylan. The work of Meek and Adams on wheat straw, in addition to that of other workers on xylans from different sources, suggests that arabinose side-chains definitely occur. Bishop and Whitaker (89) have supplied further evidence in favour of this view. They digested wheat
straw xylan with an enzyme from the mould *Myrothecium verrucaria* which hydrolysed the β 1-4 glycosidic link between xylose residues. From the enzymic hydrolysate they obtained oligosaccharides in which it was shown the following residues were present: 2X; 2X + A; 3X + A; 3X + 2A; 5X + A; and 6X + A.

It will be seen from the above that all recent workers on wheat straw xylans have concluded that uronic acid residues are structural components. It is, therefore, strange that Ehrenthal, Montgomery, and Smith (90,91), using apparently conventional isolation and purification techniques, should have found no trace of any such residues. Moreover, they found 2:6-di-O-methyl-D-glucose in the hydrolysate of their methylated xylan. They advanced the following formula for the xylan:

\[
\begin{align*}
\text{P}_1 &\rightarrow (4\text{Xp})_m \\
&\rightarrow (4\text{Xp})_y \\
&\rightarrow (4\text{Xp})_x \\
&\rightarrow \ldots
\end{align*}
\]

Where \( \text{P}_1 \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)

\( \rightarrow (4\text{Xp})_x \)

\( \rightarrow \ldots \)

\( \text{Afl} \rightarrow (4\text{Xp})_m \)

\( \rightarrow (4\text{Xp})_y \)
Their conclusion that corn-cob xylan had a similar structure is astonishing since 2:6-di-O-methyl-D-glucose has never hitherto been noted in the hydrolysates of methylated xylans. The possibility exists that it may have been derived from an associated methylated glucosan or glucopentosan. This seems unlikely, since no trimethyl glucose was obtained in the hydrolysate and it would therefore be necessary to postulate a multibranched structure to account for the dimethyl glucose.

Much other work has been carried out recently on wheat straw. Harwood (92) has investigated the action of acid-chlorite on it (see p.10). Bishop (93) has obtained crystalline xylans from wheat, barley, and rye straw hemicelluloses by autoclaving them with distilled water. The xylans so obtained contained only xylose residues. Bishop, by viscometric and periodate oxidation studies, determined that the degree of polymerisation of the parent wheat hemicellulose was 150 compared to 33 for that of the crystalline degraded xylan obtained from it. If a branch-point existed in a xylan chain then the xylose residue on which the branching occurred would not be attacked by periodate and xylose should then be detectable in the hydrolysate of the oxidised xylan. Conversely the absence of a branch point will lead to the absence of xylose in such a hydrolysate. Periodate oxidation studies indicated that the crystalline xylans obtained from wheat,
rye and barley hemicelluloses were unbranched.

**Wheat Leaf.**

Adams (94) extracted the hemicelluloses from the acid-chlorite holocellulose of wheat leaf by treating it, in an atmosphere of nitrogen, with alkali. The arabinoxylan could be represented by the following formula:

\[
Xp_1 \rightarrow [(\text{Xp}_1)_a \rightarrow \text{Xp}_1 \rightarrow (\text{Xp}_1)_b]_n \rightarrow \text{Xp}_1 \rightarrow [(\text{Xp}_1)_a \rightarrow \text{Xp}_1 \rightarrow (\text{Xp}_1)_b]_m \rightarrow \text{Xp}
\]

\[
\frac{3}{1} \quad \frac{3}{1} \quad \frac{3}{1}
\]

\[
\text{Af} \quad \text{GpA} \quad \text{Af}
\]

\[a+b = 9, \quad n \text{ or } m = 0, 1, 2, \text{ or } 3, \quad n+m = 3, \quad \text{GpA} = \text{glucuronic acid residue}, \quad \text{Xp} = \text{xylopyranose residue}, \quad \text{Af} = \text{arabofuranose residue}.
\]

**Wheat bran.**

Adams (95) recently reported details of work carried out on an arabinoxylan isolated from 'bee-wing' wheat bran. The hemicelluloses were extracted with alkali under nitrogen. Adams endeavoured to preferentially remove a possibly-occurring arabinose-rich fraction by treating the crude hemicelluloses with 70% aqueous ethanol, but he failed to observe any difference between the hydrolysate of the extracted material and that of the residue.
hemicelluloses did not form an insoluble copper-complex. He obtained no evidence of the existence of a mixture, when, following acetylation, he fractionated the product from chloroform by the addition of light petroleum. He therefore concluded that he was dealing with a homogeneous material and found that therein the ratio of arabinose to xylose residues was 1.0 : 0.78.

After deacetylation he methylated the araboxylan and then hydrolysed the product. The following molecules were found in the hydrolysate:

- Methylated uronic acid (7 parts); D-xylose (3 parts);
- 2-α-methyl-D-xylose (4 parts); mono-α-methyl-L-arabinose (3 parts);
- 2:3-di-α-methyl-D-xylose (4 parts);
- 2:5-di-α-methyl-L-arabinose (7 parts);
- 2:3:4-tri-α-methyl-D-xylose (5 parts);
- 2:3:5-tri-α-methyl-L-arabinose (6 parts).

From the rotation of the mono-α-methyl-L-arabinose fraction and from the results of its oxidation with lead tetraacetate, Adams concluded that both 3 and 5 mono-α-methyl-L-arabinoses were present; this conclusion is suspect. He has not as yet investigated the acid fraction.

The occurrence of the above methylated sugars indicated that in the araboxylan some arabinose residues were present as side-chains, some were linked through their 1 and 3
positions, while others were linked through their 1, 2, and 5 or their 1, 2 and 3 positions. This indicated that some of the arabinose residues occurred in the core of the xylan and this view was supported by the fact that mild acid hydrolysis led to the scission of only 65% of the arabinose residues. The fact that the araboxylan, unlike the xylan from wheat straw, was not attacked either before or after removal of the arabinose chains, by an enzyme preparation from the mould *Myrothecium verrucaria* indicated that the structure was in some way fundamentally different from that of the straw xylan (p. 28).

**Wheat flour.**

*Perlin* (59,80) treated a commercial wheat flour with water at 15°. Part of the material dissolved and was precipitated by the addition of alcohol to the solution. Acetylation of this material, under anhydrous conditions, was followed by the fractional precipitation of the resultant acetates from chloroform solution by the addition of light petroleum. Alkaline deacetylation yielded a product the hydrolysate of which was shown to be 99% pentose. Methylation studies on this fraction led *Perlin* to advance the following basic structural repeating
unit for the wheat araboxylan.

\[
\begin{array}{c}
\text{Af} \\
1 \\
\downarrow \\
3 \\
\text{Xp} \\
2 \\
\uparrow \\
1 \\
\text{Af}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Af} \\
1 \\
\downarrow \\
3 \\
\text{Xp} \\
2 \\
\uparrow \\
1 \\
\text{Af}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Xp} \\
3 \text{Xp}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Xp} \\
4 \text{Xp}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Xp} \\
4 \text{Xp}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Xp} \\
4 \text{Xp}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Xp} \\
4 \text{Xp}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Xp}
\end{array}
\Rightarrow
\begin{array}{c}
60 \text{ such units in the molecule.}
\end{array}

\text{Af} = \text{arabofuranose residue.}
\text{Xp} = \text{xylopyranose residue.}

He noted that progressive removal, by gentle acid hydrolysis, of the arabinose side-chains led to a decrease in the solubility of the araboxylan in water and ultimately to a xylan, akin to those in straws and woods, which was insoluble in water but soluble, with some difficulty, in dilute alkali. He put forward the interesting hypothesis that the addition or removal of arabinose side-chains could be the mechanism by which the plant ensures the transport or deposition, respectively, of the pentosan. If the hypothesis were correct then the water-soluble pentosans in the grain may be related to the water-insoluble pentosans and polyuronides to be found elsewhere in the plant tissue. The
presence of uronic acid residues in the straw, leaf and bran of wheat and their absence from the grain, indicates that the inter-relationship is not quite as simple as that suggested.

The above structures for wheat hemicelluloses are typical in their diversity of those found, not only in annual plants, but also in woods.

**Esparto grass.**

Chanda, Hirst, Jones, and Percival (96) have reported the isolation of a xylan containing only xylose residues from esparto grass (*Stipa tenacissima* L).

Extraction of the hemicelluloses was effected by treating the grass holocellulose with dilute alkali. A hydrolysate of the crude hemicellulosic material was found to contain arabinose (7.5%), glucose (5.7%), and xylose (8.1%). The formation and subsequent decomposition of a series of copper complexes was followed by the treatment of the hemicellulosic material with hot water. A product was obtained which on acid hydrolysis gave only xylose. Shrenthal, Montgomery, and Smith (90) repeated this work and obtained a similar xylan. Chanda *et al.* methylated and hydrolysed the xylan and separated the resultant syrup into its
individual components by eluting them from a cellulose column - the first time that this method of separation had been used to aid the investigation of a hemicellulose.

From the amount of tri-\text{-}O\text{-}methyl\text{-}D\text{-}xylose they concluded that there was one non-reducing group for every 35 \( \pm \) 3 residues. By measurement of the osmotic pressure of a solution of the xylan they determined the degree of polymerisation to be 70 and they advanced the following structure for the molecule:

\[
\begin{align*}
X\text{pl} &\rightarrow (4\text{Xpl})_a \rightarrow 4\text{Xpl} \rightarrow (4\text{Xpl})_b \rightarrow 4\text{Xpl} \\
&\uparrow \\
X\text{pl} &\rightarrow (4\text{Xpl})_c \rightarrow 4\text{Xp} \\
\end{align*}
\]

\[a + b + c = 70 \pm 5\]

The amount of formic acid liberated from the end groups on periodate oxidation (p. 69) is in agreement with this structure.

Aspinall, Hirst, Moody, and Percival (97) also isolated the hemicelluloses of esparto grass by the method of Chanda \textit{et al.}. Treatment of the hemicelluloses with 70\% aqueous ethanol led to the removal of an arabinose-rich fraction which on hydrolysis yielded
xylose (12 parts), arabinose (5 parts), glucose (1 part), and galactose (1 part). From methylation studies they concluded that there was either a mixed polysaccharide present containing xylose, arabinose and galactose residues or there were two polysaccharides of the following types:

\[
\cdots \cdots \rightarrow 4\text{Xpl} \rightarrow 4\text{Xpl} \rightarrow (4\text{Xpl})_2 \rightarrow 4\text{Xpl} \rightarrow (4\text{Xpl})_2 \rightarrow \cdots \cdots
\]

\[
\begin{array}{ccc}
& & \uparrow \\
& & 1 \\
& & \text{Af} \\
\end{array}
\]

\[
\cdots \cdots \rightarrow 3\text{Gal pl} \rightarrow 3\text{Gal pl} \rightarrow 3\text{Gal pl} \rightarrow \cdots \\
\begin{array}{ccc}
& & \uparrow \\
& & 1 \\
& & \text{Gal p} \\
\end{array}
\]

\[Xp = \text{xylopyranose residue.}\]
\[Af = \text{arabofuranose residue.}\]
\[\text{Gal p} = \text{galactopyranose residue.}\]

Moody et al. found a small amount of 2,3-di-O-methyl-L-arabinose in the hydrolysate of the methylated hemicellulose(s); this they concluded was due to under-methylation and not to the presence of an araban.

Flax straw.

McIlroy (93) investigated a polyuronide from N. Z. flax
(Phorium tenax). Normally hydrolysis of a methylated xylopolyuronide yields, amongst other products, a methylated aldobiuronic acid of type I, but he obtained instead a methylated aldotriuronic acid of type II which indicated that the xylan had structure III. In this case it will be seen that the uronic acid residue is not, as in the molecules hitherto considered, attached directly to the main xylan chain, but to it through an anhydro-xylose unit.

![Chemical structures](image)

\[Xp_{1a} \rightarrow 4Xp_{1} \rightarrow 4Xp_{b}\]

\[(III) \quad a + b = 18-20\]

GpA = glucopyranose residue

GpA \quad Xp = xylopyranose residue.
Geerdes, and Smith (135) have recently reported the isolation of a different xylan from flax straw. It contained L-rhamnose and D-glucuronic acid residues. Rhamnose had previously been found by Weiting (99) in the hydrolysate of a flax straw xylan. It has also been found in the hydrolysates of beech (100), aspen (106), and spruce (109) woods.

Corn.

The results of a structural investigation of a xylan isolated from corn cobs by Ehrenthal, Montgomery, and Smith (90) have already been mentioned (p. 29).

Whistler, and Durso (101) have shown that it is possible to separate a mixture of oligosaccharides by adsorbing them on a column of charcoal and celite and then desorbing them and eluting them from the column by washing it with various concentrations of aqueous alcohol. Increasing concentration of ethanol leads to the desorption of oligosaccharides of higher molecular weights.

Utilising this method Whistler and co-workers (102, 103) have separated a series of xylodextrins obtained by partial hydrolysis of corn cob xylan. They have isolated oligosaccharides in the crystalline form containing from two to eight xylose residues. They
have concluded that these molecules are composed of linear chains of \(\beta\)-D-xylopyranoside units linked 1\(\rightarrow\)4. They have shown that if the degree of polymerisation is plotted against the melting point then a smooth curve is obtained in which the difference between members grows successively smaller with increasing degree of polymerisation.

Uronic acid residues normally appear to be linked to xylose residues either 1\(\rightarrow\)2 or 1\(\rightarrow\)3. Whistler, and Hough (104) have recently found that part of the uronic acid residues present in hemicellulose B from corn cobs appears to be linked either 1\(\rightarrow\)4 or 1\(\rightarrow\)5; it is considered more likely to be the former.

Weihe, and Phillips (105) investigated a xylan from cornstalks and they concluded that in it uronic acid, arabinose, and xylose residues were present in the ratio of 2 : 7 : 19.

**Pear cell-wall.**

Chanda, Hirst, and Percival (39) extracted a xylan from delignified pear-cell-wall material by treating it with 24% aqueous potassium hydroxide. From methylation
studies they ascribed the following structure to the molecule:

\[
Xpl \rightarrow (4Xpl)_a \rightarrow 4Xpl \rightarrow (4Xpl)_b \rightarrow 4Xpl \rightarrow (4Xpl)_c \rightarrow 4Xp \\
\downarrow 3 \uparrow 3 \\
\downarrow 1 \uparrow 1 \\
Xpl \rightarrow (4Xp)_d \quad \text{GpA}
\]

\[a + b + c + d = 110 \pm 5.\]

\[Xp = \text{xylopyranose residue.}\]

\[\text{GpA} = \text{glucuronic acid residue.}\]

Aspinall, Hirst, and Mahomed (100) have investigated a xylan extracted from a non-delignified beechwood (Fagus sylvatica) by McDonald (152). They concluded that it could be represented by the following formula:

\[
Xpl \rightarrow [ (4Xpl)_a \rightarrow 4Xpl \rightarrow (4Xpl)_b ]_7 \rightarrow 4Xp \\
\downarrow 8 \uparrow 1 \\
\downarrow \text{GpA}
\]

\[a + b = 9\]

\[Xp = \text{xylopyranose residue.}\]

\[\text{GpA} = \text{glucuronic acid residue.}\]

Mahomed et al. showed that the beech wood xylan
contained 4-O-methyl-D-glucuronic acid residues. Residues of this type have been found in many woods: aspen (Populus tremuloides) (106), *Eucalyptus regnans* (107, 108), scots pine (*Pinus sylvestris*) (109), black spruce (*Picea nigra*) (109), and birch (48). In all cases, excepting possibly *Eucalyptus regnans* which has not been fully investigated, the anhydride has been found linked to the C(2) position of a xylose residue.

**Crystalline, degraded xylans.**

Many crystalline degraded xylans have in recent years been prepared. The work of Bishop (93) on wheat, barley, and rye degraded xylans has already been mentioned (p.29). Crystalline xylans were first prepared by Yundt (111) working with partly depolymerised barley straw and birch wood xylans. Yundt obtained the xylan crystals in two forms (hexagonal and sphero-crystals) while more recently Roelofsen, working with a rye straw xylan, has obtained crystals of a new form (110).

**Seaweed polysaccharides.**

Chanda, and Percival (112), and independently, Barry and co-workers (113), have investigated a non-hemicellulosic xylan extracted from the red seaweed *Rhodymenia palmata*. They extracted the xylan with dilute hydrochloric acid. Examination of the products of hydrolysis of
methylated xylan led the former workers to the conclusion that in the xylan the xylose residues are linked one to the other in some cases by 1→3 and in other cases by 1→4 glycosidic bonds.

**Polysaccharides containing arabinose residues.**

Although arabinose is known to occur in nature in both the D (114) and the L forms only the latter form has been encountered in plants. Until recently it was believed that arabinose occurred exclusively in plants in the furanoid form; a view which was proved erroneous by the discovery of arabopyranose residues in sapote gum (115), and in an arabogalactan from larch wood (116). In all other cases, as far as is known, L-arabofuranose residues alone are found. In addition to their occurrence in araboxylans they have been found in gums, and mucilages (117) and in seaweed polysaccharides (112,113). Arabans (118) are members of the pectic triad which consists of a closely associated group of polysaccharides which includes also galactans and polygalacturonic acids. Pectic materials (119) are known to occur in the middle lamella of plant tissues and in the primary cell walls and to a smaller extent in the secondary walls.
As was mentioned earlier, uncertainty often surrounds the discovery of arabinose in hemicellulosic hydrolysates (95). The question of whether or not the arabinose is a derivative of an araban or of a mixed cellulosan or polyuronide is sometimes uncertain. Where the hydrolysate of methylated hemicelluloses is found to contain only trimethyl-arabinose, in addition to other methylated hexoses and non-arabinose pentoses, it must be concluded that no araban was present, and that the arabinose must have been derived from residues attached either as side-groups or as end-groups in a cellulosan or polyuronide. So far all pure arabans investigated have been obtained from pectic materials. There is little evidence to uphold the view that hemicellulose preparations may be contaminated with arabans.

Hirst, and Jones (120) investigated a peanut araban and suggested the following as a possible formula for it:
The Action of Enzymes on Xylan, (123).

Enzymes able to catalyse the hydrolysis of xylans are widely distributed in nature. Xylanase activity has been noted in extracts from germinating barley (134, 122), from seaweeds (123), from termites (124), from vineyard snails (126, 122), from insect larvae (125), and from the intestines of many higher animals (126). Enzyme preparations with xylanase activity have been isolated from many soil and compost micro-organisms of the Aspergillus species [A. oryzae (127), A. niger (111), A. foetidus (129), A. terreus (130), A. trichoderma (130), and A. lignorum (130)] and from individual members of the Cellvibrio, Micromonospora, Streptomyces (131), and Penicillium (130) species. Xylans used as substrates have included those from woods [beech (127), birch (111), poplar (125), and oak (75, 76)], straws [rice (126), and wheat (133)], pit shells [cherry, and plum (122)], rushes (126), elderberry pith (124), and corn cobs (129).

Early work of O'Dwyer (75, 76), Voss and Butter (122), and of Grassmann (127) has been adequately reviewed elsewhere (128) and here it is intended to review only the more recent work on xylanases.

Sørensen (131,133) while investigating the soil mould Chaetomium globosum found that a dried mycelium powder
and a cell-free extract from the organism possessed xylanase activity. This activity was much greater in the case of a mycelium prepared from an organism grown on a basal medium containing xylan than in an mycelium prepared from an organism grown on a medium containing material other than xylan as source of carbon. This indicated that the xylanase in the Chaetomium globosum was an adaptive enzyme and moreover it was found that an adaptive mannanase and an adaptive cellulase were also present in the dried mycelium. Since the activity of those enzymes was not enhanced by growth of the organism on a xylan substrate, it was deduced that the enzyme responsible for the xylanase activity of the mycelium was distinct from those responsible for the mannanase and cellulase activities.

Sørensen found that enzymic hydrolysis of wheat straw xylan yielded xylose, arabinose, and a water-soluble enzyme-resistant polysaccharide; this latter, it was shown by chromatographic examination of an acid hydrolysate, contained xylose, arabinose, and glucose residues (ca. 1:1:1) and an amount of an uronic anhydride. He noted that while culture filtrates of the Chaetomium globosum catalysed the hydrolysis of xylotriose to only xylose and xylobiose, cell materials from the same
source effected the hydrolysis of the xylobiose. On the basis of this and other evidence Sørensen has concluded that the organism produced two enzymes with different xylan hydrolytic functions.

In other work Sørensen (131) noted that cultures of a Streptomyces sp. and of a Micromonospora sp. from compost appeared to contain two enzymes active in the hydrolysis of xylan and with properties similar to those noted in the enzyme preparations from Chaetomium globosum.

Work by Bishop and Whitaker (89) has already been outlined in which an enzyme preparation from Myrothecium verrucaria was used to catalyse the hydrolysis of wheat straw xylan (p.27).

Recently Whistler and Masak (129) have hydrolysed corn cob xylan using an enzyme preparation from Aspergillus foetidus. They fractionated their enzyme preparation on columns of cation-exchange resins, of celite, and of carboxymethylcellulose. They obtained two fractions one of which catalysed the hydrolysis of the corn-cob xylan but not of the corn-cob 'Hemicellulose B' (a xylan) while the other catalysed the hydrolysis of the Hemicellulose B but not the xylan.
Introduction to the Present Work.

In the course of the work carried out by the author the structure has been determined of a xylan extracted from oat straw holocellulose. Attempts have been made to isolate, from the same source, another hemicellulose containing a higher percentage of arabinose residues than did the above xylan. A study has also been made of the action of various enzyme preparations, possessing xylanase activity, on the above oat straw xylan and on other xylans.

There appears to have been but little investigation of the hemicelluloses of oat straw. Allen and Tollens (164) in 1890, and Hébert (165) in 1892 extracted a 'straw-gum' from oat straw by treating the straw with alkali. Norman (166) fractionated oat-straw hemicelluloses into two fractions A and B. He determined that Hemicellulose A contained 10.6% of uronic anhydride and 63.0% of anhydropentose whilst Hemicellulose B contained 31.8% of the former and 56.5% of the latter.
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In the Introduction reference has been made to various experimental techniques of particular or of historic importance. It is proposed to deal in the following sections exclusively with the experimental methods that have been used in the course of the present work.

**Paper Chromatography** (136).

**Qualitative.**

Separations were carried out by the method of Hirst, Hough, and Jones (137). Chromatogram papers (12 x 40-60 cm.) were generally cut from Whatman No. 1 paper but on some occasions Nos. 3 or 3MM were used. After irrigation of papers on which sugars had been spotted the papers were dried in an oven at 110° and were then lightly sprayed with one of the reagents listed below. The papers were replaced in the oven until spots appeared; this is subsequently termed the development stage (it should be noted that certain chromatographic workers use this term in a different sense). The colours of spots were examined in daylight and in the light of a Hanovia ultra-violet lamp. Where possible standard sugars were chosen so as to duplicate the sugars present in the sugar solution being investigated.
Sprays.

1. Saturated aqueous solution of aniline oxalate.
2. 3% acetic acid solution saturated with aniline oxalate.
3. 10% acetic acid solution saturated with aniline oxalate.
4. 3% solution of p-anisidine hydrochloride in n-butanol.

Irrigants. Papers were irrigated with one of the following solvent mixtures. Irrigants Nos. 1-4 and 6 consisted of two phases and the organic-rich phase was used for irrigation while the aqueous phase was placed, in a beaker, on the bottom of the chromatogram tank.

2. n-butanol/acetic acid/water (40:10:50).
3. ethyl acetate/pyridine/water (10:4:3).
4. benzene/n-butanol/pyridine/water (1:5:5:3).
5. methyl ethyl ketone half-saturated with water.

Where it is left unspecified in the subsequent text it may be assumed that Irrigant No. 4 and Spray No. 1 were used.

Quantitative.

Quantitative separations were carried out by the method of Flood, First, and Jones (139). The same chromatographic
papers, sprays, and irrigants were used as in the qualitative work.

Sugars were generally eluted from papers by the method of Laidlaw and Reid (140). The papers were hung vertically and were irrigated slowly by cold water introduced at the top of the paper from a capillary. After thorough elution the papers were dried then sprayed with Spray No. 1 and heated at 110°; they were then examined for evidence of the incomplete elution of reducing sugars. Areas of paper equal to those carrying the sugars were also eluted and the eluates were used for blank determinations.

**Hydrolysis of hemicelluloses for use in qualitative and quantitative chromatographic work.**

For quantitative work a weighed sample (ca. 20 mg.) of the hemicellulose was heated (6 h; 100°) in a sealed tube with sulphuric acid (1 ml.; N). After cooling the hydrolysate, it was neutralised by the addition of barium carbonate; the barium residues were centrifuged down. If it were wished to determine actual quantities, as opposed to ratios, of the sugars then a known weight (ca. 10 mg.) of a reference sugar (L-rhamnose) was added prior to neutralisation.

Hydrolysates for qualitative work were prepared in the same way without accurate weighing or pipetting.
Rough quantitative estimations were made by visual inspection of the intensity of the spots produced by different sugars on developing suitably sprayed papers. This method was found to be quite accurate for the estimation of sugars present in the ratio of 1:5 or less. A more accurate modification is detailed later (p.119).

Paper Ionophoresis.

Ionophoretic separations were carried out by a modification of the method of Consden and Stanier (141). Whatman No. 1 paper (12 x 34 cm.) was used and on it a line was drawn 15 cm. from the end later to be placed near the cathode. The paper was dipped in 0.05M aqueous borax solution and was then evenly blotted. The wet paper was placed in a glass frame and the ends of the paper were dipped into external electrode-compartment containing the same buffer solution; and the levels of the liquids in the compartments were equilibrated to prevent capillary siphoning through the paper. A single fairly concentrated spot of a sugar solution was placed on the line on the paper and standard sugars were similarly placed at other positions on the line. The developing-dish in which the frame was situated was filled to above the level of the paper with chlorobenzene. A potential difference of 450 volts was applied between the
ends of the paper and was maintained for periods ranging from \( \frac{1}{2} \) - 8 hours; 4 hours was generally found to be most suitable. After drying, the paper was sprayed with Spray No. 1 (slightly acidified by the addition of a little acetic acid) and spots were developed by heating the paper at 110°.

In work on the enzymes the papers (Whatman Nos. 1, 3, and 3MM) were impregnated with various buffer solutions; details are given later. Chlorobenzene was not used; the papers were clamped instead between glass plates (142) which had been lightly coated with silicone grease. The potential difference used varied from 250-1,200 volts and the duration of its application from an hour to two days. Staining techniques, to be outlined later, replaced the spraying techniques.

Quantitative Determinations.

**Micro-determinations of methylated aldoses using sodium hypoiodite (137).**

An 0.1N solution of iodine (1 ml.) was added from an "Agla" micrometer syringe to the sugar solution (5 ml.) in a boiling tube (75 ml.) fitted with a B 24 ground glass stopper. Into the tube there was pipetted a solution (2 ml.) of 0.2M sodium carbonate and the tube was closed with a stopper moistened with aqueous potassium iodide (10%).
A control determination was carried out on 5 ml. of water. After standing for 2\frac{1}{2} hours at room temperature the solutions were diluted to 25 ml. and sulphuric acid (20 ml.; 2N) was added. The solutions were titrated with sodium thiosulphate solution (0.01N).

**Micro-determinations of sugars with sodium metaperiodate (143).**

To the sugar solution (ca. 7 ml.), in a boiling tube (75 ml.), there was added sodium metaperiodate solution (2 ml.; ca. 0.25M) and the resultant solution was heated for 20 min. on a boiling water-bath; during this time the upper part of the tube was jacketed in a spiral tube through which cold water was passed. The solution was cooled under the tap and neutral ethylene glycol (ca. 0.2 ml.) was added to destroy the excess of periodate. The formic acid released was determined by titration with 0.01N sodium hydroxide; screened methyl red was used as indicator. The end-point of the titration was determined by comparison of the colour of the sugar reaction-mixture with that of a solution containing only water, ethylene glycol and indicator.

**Schaffer-Hartmann determinations of reducing values (144).**

Reducing values of sugars in enzymic digests were
determined by a modification of the method of Stiles (145) which is itself a modification of the method of Schaffer and Hartmann.

Preparation of the reagents.

Reagent A. Anhydrous sodium carbonate (40.0 g.), copper sulphate (5.0 g.), tartaric acid (7.5 g.), potassium iodide (10.0 g.), potassium iodate (0.7 g.), and dipotassium oxalate (18.4 g.).

Reagent B. Sodium thiosulphate (25 g.) and sodium hydroxide (1 g.).

Reagent A was prepared by dissolving the first three reagents in water (250 ml.) and adding the solution to a solution of the potassium salts in water (250 ml.). Reagent B was prepared by dissolving the reagents in water and making the volume of the solution up to a litre.

Reagent A (2-10 ml.) was pipetted into a sample (1-5 ml.) of the enzymic digest in a boiling-tube (75 ml.). The solution after being heated on a boiling-water bath for 20 minutes was cooled and sulphuric acid (10 ml.; \( \sqrt{W} \)) was added to it. The tube was stoppered and left for 10 minutes and the contents were then titrated with Reagent B (ca. 0.005\( \sqrt{W} \)). A number of determinations were made of the reducing values of solutions containing known weights of xylose and a curve was plotted relating the weight of xylose to the volume of titrant.

56
Methoxyl determinations. (151).

Methoxyl determinations were carried out by a modification of the method of Vieböck and Brecher (146). The sample (3-5 mg.), together with a few crystals of phenol, was digested with distilled hydriodic acid (2 ml.) and the volatile alkyl iodide formed was swept over by a stream of carbon dioxide into a 10% solution of sodium acetate in glacial acetic acid (6 ml.) to which there had been added a little bromine (ca. 6 drops). The excess of bromine was removed by treatment with formic acid. Sulphuric acid (ca. 5 ml.; 2N) and crystalline potassium iodide (ca. 0.5 g.) were added to the acetic acid solution and it was then titrated with sodium thiosulphate (0.05N). Potential contaminants carried with the alkyl iodide were removed by bubbling the vapour through a spray-trap containing aqueous solutions of cadmium sulphate (1 ml.; 5%) and sodium thiosulphate (1 ml.; 5%).

Uronic anhydride determinations.

Uronic anhydride was determined by the method of McCready, Swenson, and Maclay (147). The sample (ca. 0.2 g.) was heated with hydrochloric acid (30 ml.; 19%) in a flask (150 ml.) on an oil-bath (145°C). The carbon dioxide as it was evolved was swept along in
a stream of dry air, from which carbon dioxide had been previously removed, and was bubbled upwards through an adsorption tower containing glass beads and carbonate-free sodium hydroxide solution (0.1N; 25 ml.). A little butanol (5 drops) was added to the tower to prevent frothing. The air was drawn through at a steady, controlled rate for two hours. Heating was discontinued and to the alkali there was added barium chloride solution (10 ml.; 10%). The alkali was titrated with hydrochloric acid (0.1N) against phenolphthalein. Blanks were run under identical conditions on the reagents and, separately, on xylose.

**Lignin determinations.**

Lignin was determined by the method of Mahood and Cable (148). The dried sample (0.5-1.0 g.) was mixed with ten times its weight of 72% sulphuric acid. After 15 hours at room temperature the acid was diluted to 3%. The mixture was boiled for 2 hours then the residue was recovered on a sintered glass funnel. The funnel and contents were dried (105°) to constant weight.

**Ash determinations.**

Samples were ignited on their own and then subsequently re-ignited with a few drops of concentrated sulphuric acid.
Melting-Points (149).

Melting-points were determined on a Kofler hot-stage melting-point apparatus.

X-ray Powder-Diffraction Photographs.

The finely powdered sample of crystalline material to be photographed was introduced into a fine-walled glass capillary and this was placed in position in a Debye-Scherrer 9 cm. X-ray powder-diffraction camera. The capillary was rotated in the path of a beam of nickel-screened K-α radiation from a copper anticathode; the voltage across the tube was 50 kV. The period of irradiation varied.

Purification of Solvents.

Solvents for use in chromatography were purified by the standard procedures outlined by Vogel (150). Butanol was purified by heating it under reflux over sodium hydroxide (40 g./l.) for 3 hours. The solvent was then distilled (132).
A Xylan From Oat Straw

EXPERIMENTAL

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EXPERIMENTAL

The straw (variety Sun II) was grown at Easter Howgate and was cut in late September, 1952.

The heads of the straws and all withered parts were removed and the straws, cut in short lengths (ca. 5 cm.), were reduced to a fine, fibrous, non-powdery product by passing them through the 1/8 in. mesh of a Raymond hammer-mill.

Delignification of the straw and extraction of the hemicelluloses on a small scale.

For details of the methods see the following two sections.

A sample of the milled straw was dried (120°; 6 h) (moisture, 18.0%). The straw was delignified and the resultant holocellulose was thoroughly washed and dried (yield, 80% on the weight of dry straw). The holocellulose was treated with sodium hydroxide solution (4%) and the hemicelluloses were precipitated from the acidified solution by the addition of acetone [yield of hemicelluloses, 3.1 g. (19%, on the weight of dry straw)].

Delignification of the straw on a large scale.

The milled straw was delignified by a modification of a method of Wise (22). The straw (365 g.) was suspended in a mixture of water (6,600 ml.) and glacial acetic acid (660 ml.), and to it there were added crystalline sodium
acetate (77 g.), and sodium chlorite (1,850 g.). The reaction was carried out in a number of flasks (51.) placed in a water-bath (30°). Within 15 min. the temperature of the reaction-mixture had risen to 50-60° and ice was added. The reaction was thereafter conducted at 30°; the flasks were frequently and vigorously shaken. The concentration of the chlorine dioxide in the gaseous phase was reduced, in order to minimise the danger of explosion, by partly removing the gas under suction. Throughout the reaction the pH remained constant at 5-6. After 24 hours the brown liquor, resulting from the reaction, was removed by filtration and the residue washed thoroughly with ice-cold water and then with acetone. The residue was dried under suction in a Buchner funnel. The product (holocellulose) was practically colourless; it retained the fibrous structure of the straw.

Extraction of the hemicelluloses.

Part of the above holocellulose was placed in a stoneware jar (3.5 l.) together with a quantity of glass marbles. The jar was filled to the neck with a cold 4% aqueous solution of sodium hydroxide and nitrogen was bubbled through the slurry to remove dissolved oxygen. The jar, thoroughly sealed by coating the bung liberally with paraffin wax, was rolled mechanically for 12-15 hours to macerate the holocellulose. The hemicelluloses were extracted by treatment of successive portions of the holocellulose in this way; a total volume of
11 litres of alkali was used. The resultant slurry was filtered through muslin then through Whatman No. 1 paper. The brown filtrate was mildly acidified with acetic acid; after a few minutes a slight precipitate (A) was formed and was removed by centrifugation. To the centrifugate there was added rather more than 50% (by original volume of the centrifugate) of acetone. A flocculent white precipitate (B) was formed and was separated at the centrifuge. It was washed successively, several times, with: aqueous acetone (1:1), then aqueous alcohol of increasing concentration, then alcohol, and finally with dry ether; the product was dried on a porous plate in air and was then powdered. Slight traces of ether made dissolution of the hemicelluloses in alkali difficult and the product was, therefore, dried in a vacuum desiccator over potassium hydroxide (yield, 46 g.).

The small amount of hemicellulosic material which was extracted by a second alkaline treatment of the holocellulose was rejected.

Examination of hemicelluloses A and B.

Samples (ca. 20 mg.) of hemicelluloses A and B were separately hydrolysed with sulphuric acid (2 ml., IN; 6 h. at 100°). The hydrolysates were examined chromatographically; sugars corresponding in Rf values to the standards xylose, and arabinose were major constituents and there were traces corresponding to glucose and galactose. The only significant difference between the two hydrolysates was that that of
hemicellulose A appeared to contain slightly more glucose. Paper-chromatographic separations of portions of the hydrolysates were followed by quantitative estimations of sugars by determination of the formic acid released on periodate oxidation (p. 55).

Hemicellulose A: xylose 94.5%, arabinose 5.5%.
Hemicellulose B: xylose 94.3%, arabinose 5.7%.
(calculated as 100% pentose).

As fractions A (2 g.) and B (44 g.) were chromatographically similar they were combined. The combined fraction had 
$\left[\alpha\right]_D^{12} -94.7^\circ (c, 0.6 \text{ in } N \text{NaOH}).$

Fractionation of Hemicelluloses

The finely powdered xylan (48 g.) was steeped overnight in water (2 l.) and to it there was added aqueous sodium hydroxide solution (1,400 ml., 10%). The addition of an excess of Fehling's solution produced a copious, blue, gelatinous precipitate. The gel was separated by centrifugation, dispersed in water (2.5 l.), and the xylan regenerated by adding hydrochloric acid (2N) to give a normal solution. The copper-complex appeared to decompose completely within a few minutes and after a short time a slight white precipitate formed. The precipitate was centrifuged down and the centrifugate treated with acetone as before to precipitate the remaining hemicelluloses. Chromatographic
examination of hydrolysates of the two precipitates indicated that they were indistinguishable and they were accordingly combined. In subsequent decompositions of copper-complexes it was noted that on some occasions (as above) a precipitate formed after acidification and prior to the addition of acetone; in other cases it did not. The effect appeared to depend principally on the hemicellulosic concentration, although the pH may have had an influence.

The precipitates formed on copper-complex decompositions were greenish-blue and, in an endeavour to remove what were believed to be adsorbed copper ions, the precipitate was washed with a mixture of acetone, water and 2N hydrochloric acid (50:45:5). The colour was extremely persistent, but after seven washings a white precipitate was obtained. This product is called xylan 1 and subsequent products obtained after copper-complex decompositions are systematically numbered. Later copper-complexes were decomposed by making the solution 0.5N with respect to the acid and by extending the period of treatment to half an hour; it was then found that the precipitate produced on the addition of acetone was only slightly contaminated with copper which was readily removable. It appears probable that the difficulty previously experienced in the removal of copper was due to the incomplete decomposition of the copper-complex and not to adsorbed copper ions.

The formation and decomposition of a further four complexes
was followed by the precipitation of xylans 2-5. With the exception of xylan 3 all products were dried to facilitate their dissolution in alkali prior to the formation of the next complex.

Yields: xylan 1 33 g. (72.3% of crude xylan).
  xylan 2 29.0 g. (87% of xylan 1).
  xylan 3 25.5 g. (88% of xylan 2).
  xylan 4 not dried.
  xylan 5 19.0 g. (74.5% of xylan 3; 41.3% of crude xylan).

Paper chromatographic separation of a hydrolysate of xylan 5 was followed by determination of the formic acid released on periodate oxidation of the sugars (Found: arabinose, 3.0%).

A sample (0.6 g.) of xylan 5 was passed through a further four copper-complex formations followed in each case by the regeneration of the xylan. The complex of xylan 5 was decomposed by treatment for 3 hours with N hydrochloric acid. Xylans 1-3 and 5-9 were hydrolysed with sulphuric acid (1 ml, N; 6 h at 100°) and the hydrolysates examined chromatographically: papers were sprayed with aniline oxalate solution and developed at 110°. There was no visually apparent reduction in the arabinose/xylose ratio of xylans 5-9 compared to xylan 3. The hydrolysates all contained traces of glucose and of galactose.

To remove any water-soluble matter that might remain,
xylan 5 was covered with water (500 ml.) and was allowed to stand overnight. The xylan was centrifuged down and dried; there was a little loss due to dispersion. Chromatographic examination of a hydrolysate of the xylan indicated that the xylan was indistinguishable from xylan 5.

Isolation of a Second Quantity of Xylan.

A second batch (660 g.) of milled straw was delignified and extracted as before (yield of crude xylan, 91 g.). Five copper-complexes were successively formed and the xylan was regenerated by the acid decomposition of each complex (yield, 42.9% of crude xylan). Chromatographic examination of a hydrolysate of this xylan indicated that the xylan was indistinguishable from xylan 5; the two quantities of xylan were accordingly combined. In all subsequent work this material was used.

It had: Ash (estimated as sulphate), 0.8%.
Ome content, 0.50%.
\([\alpha]_D^9 -95.0^\circ\) (c, 0.5 in \(\bar{N}\) NaOH).
Lignin content, 3.3%.
Uronic anhydride, 3.5%.
Periodate Oxidation Studies.

Formic acid release.

A modification of the method of Halsall, Hirst, and Jones (153) was used.

To six bottles (50 ml.) there were added: accurately weighed quantities of xylan (ca. 50 mg.), aqueous potassium chloride (2 ml.; 16%), aqueous sodium metaperiodate (5 ml.; 0.090 M), and water (5 ml.). Six further bottles were prepared in the same way with the exclusion of the addition of xylan. The bottles, securely stoppered, were shaken in the dark continuously. From time to time two bottles were withdrawn; one with, the other without, xylan. The contents of the former bottle were centrifuged and a sample (10 ml.) of the clear centrifugate was withdrawn, treated with neutral ethylene glycol (to destroy the excess of periodate) and then titrated with carbonate-free sodium hydroxide (0.01 N); methyl red was used as indicator. Control determinations were carried out on the reagents; the titres remained practically constant throughout the experiment.

<table>
<thead>
<tr>
<th>Hours from start of reaction</th>
<th>71</th>
<th>121</th>
<th>168</th>
<th>244</th>
<th>312</th>
<th>455</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles of formic acid per C₅H₉O₄</td>
<td>7.25</td>
<td>8.76</td>
<td>9.31</td>
<td>11.01</td>
<td>12.27</td>
<td>15.77</td>
</tr>
</tbody>
</table>

These results are shown graphically later (p. 114).
Periodate uptake.

To a number of clean dry bottles (50 ml.) there were added accurately weighed quantities of xylan (ca. 90 mg.), and aqueous sodium metaperiodate (20 ml.; 0.25M). Bottles were also prepared containing only sodium metaperiodate solution: these were used for control determinations. From time to time one of each of the bottles was removed, a sample of the contents withdrawn, and suspended matter centrifuged down. A portion (5 ml.) of the clear centrifugate was extracted and was diluted to 25 ml. in a graduated flask.

Determination of the periodate uptake was made by the method of Fleury and Lange (154). A sample (5 ml.) was withdrawn from the flask and to it there were added: sodium arsenite solution (35 ml.; 0.1015N), crystalline potassium iodide (1 g.), and an excess of sodium bicarbonate. After standing for 15 minutes the solution was titrated with iodine solution (0.09761N); starch indicator was used. Control determinations on the periodate were constant throughout the experiment.

<table>
<thead>
<tr>
<th>Hours from start of reaction:</th>
<th>48</th>
<th>72</th>
<th>149</th>
<th>334</th>
<th>432</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles of periodate consumed per C$_5$H$_8$O$_4$ unit.</td>
<td>0.817</td>
<td>0.834</td>
<td>0.892</td>
<td>1.003</td>
<td>1.007</td>
</tr>
</tbody>
</table>

At the end of the reaction the contents of the bottles
were combined and the resultant mixture was dialysed against tap-water until free from ions. The solution was concentrated to small volume (5 ml.) and, following the addition of sulphuric acid (5 ml.; 2N), was heated (100°; 6 h.) in a sealed tube. The hydrolysate was examined on heavily spotted chromatogram papers; a trace of a reducing sugar with the same $R_g$ value as xylose was detected.

**Methylation of Xylan.**

Finely powdered xylan (17 g.) was placed in a three-necked flask (5 l.) fitted with a dropping funnel, a vertical stirrer, and an inlet tube extending to near the bottom of the flask, and was allowed to soak in water (100 ml.) for two days. Nitrogen, freed from oxygen by bubbling through a solution of pyrogallol in aqueous sodium hydroxide, was swept through the flask and throughout this and subsequent operations a slow stream of nitrogen was maintained. Sodium hydroxide (200 ml.) was added to yield a solution 40% by weight with respect to the sodium hydroxide. After vigorous stirring for two days the xylan had not all dissolved, but the methylation was started by the slow addition of dimethyl sulphate (180 ml.) during 8 hours vigorous stirring being maintained and the reaction-mixture being cooled on ice-water. Four further methylations were carried out with the addition each time of sodium hydroxide solution (300 ml.; 40%) and, over a period of 8 hours, of
dimethyl sulphate (180 ml.). After the fifth methylation the reaction-mixture was allowed to stand for 24 hours and was then neutralised by the gradual addition of sulphuric acid (2N) to pH 8. The precipitated sodium sulphate was dissolved by heating the mixture to 90°, and the methylated xylan which then separated was removed by filtration. A sample of the methylated xylan was thoroughly washed with hot water and dried (Found: OMe, 23.3%).

A further series of five methylations was carried out in a similar way, but with the addition of acetone (300 ml.) to aid dissolution of the partially methylated xylan. After five methylations sulphuric acid (2N) was slowly added until the solution remained only slightly alkaline. The acetone was removed by distillation under reduced pressure at 30° and the pH was adjusted to 8 as before. The methylated xylan was separated from the hot solution as before and was washed with hot water to remove sodium sulphate. During washing part of the methylated xylan dispersed in the wash liquors and they were treated with chloroform to recover the material, but a singularly stable gel was formed from which no chloroform layer had separated after several days. Prolonged centrifugation separated a chloroform layer and it and a second chloroform extract were combined and the solvent removed under reduced pressure. The fractions due to dispersed and non-dispersed materials were combined and the water removed under reduced pressure at 50° to yield a methylated xylan [16.8 g. (84% yield)];
Found: OMe, 34.5%.

As difficulties had been encountered in a previous unsuccessful series of methylations using silver oxide and methyl iodide, a sample (0.7 g.) of the above methylated xylan was subjected to further methylation by heating with neutral methyl iodide and dry silver oxide. After one treatment the product (0.55 g.) had OMe, 38.6%. As no difficulty was experienced in this small scale methylation the remainder of the methylated xylan was treated in a similar way. The partially methylated xylan was heated under reflux with methyl iodide (500 ml.; previously heated with silver oxide until neutral), but the gradual addition of a small quantity of dry methanol (18 ml.) was required to effect complete solution. Silver oxide was then added in small amounts (40 x 6.2 g.) during 10 hours to the boiling methyl iodide solution which was kept in the dark and carefully tested for the possibility of developing acidity, but the solution remained neutral throughout the reaction. At the end of the reaction the methyl iodide solution was filtered, and the silver residues were treated several times with boiling chloroform to extract methylated product. The combined methyl iodide and chloroform solutions were heated with a little silver oxide to coagulate colloidal silver and the filtered solution was taken to dryness under reduced pressure. The resulting syrupy material was dissolved in chloroform (40 ml.) and the solution poured with vigorous stirring into
light petroleum (b.p. 60-65°; 600 ml.) to yield a white precipitate which was separated by centrifugation. The methylated xylan (12·6 g.) was obtained in 61·2% overall yield from the oat straw xylan (Found: OMe, 38·9%).

**Fractionation of methylated xylan.**

The methylated xylan was heated under reflux for 2 hours with solutions (150 ml.) of light petroleum (b.p. 65-70°) and chloroform ranging in volume ratios from 1:0 to 7:3. After each dissolution the solution was decanted through a small filter-paper and the paper was then returned to the flask. The residue in the flask was re-extracted either with a similar solvent mixture or with one richer in chloroform. The solvents were removed by distillation under reduced pressure at 40°.

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>No. of treatments</th>
<th>Ratio of light petroleum to chloroform</th>
<th>Appearance of product</th>
<th>OMe %</th>
<th>Wt. of fraction g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>100 : 0</td>
<td>dark-brown syrup</td>
<td>38·2</td>
<td>0·01</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>90 : 10</td>
<td>light-brown syrup</td>
<td>36·8</td>
<td>0·06</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>80 : 20</td>
<td>pale yellow semi-solid</td>
<td>37·2</td>
<td>0·45</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>75 : 25</td>
<td>white paste</td>
<td>37·8</td>
<td>4·64</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>70 : 30</td>
<td>white paste</td>
<td>38·2</td>
<td>7·45</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>residue</td>
<td>brown solid</td>
<td>-</td>
<td>0·01</td>
</tr>
</tbody>
</table>

In the subsequent text 'methylated xylan' refers to fraction 5. This product had \([\alpha]_D^{17} = -37·0°\) (c, 0·91 in CHCl₃).
Determination of the viscosity of the methylated xylan.

A solution of methylated xylan in m-cresol was prepared and the time ($T_1$) required for the flow of a known volume of this solution through an Ostwald viscometer was determined. The time ($T_2$) of flow of pure solvent was also determined and the specific viscosity ($\eta_{sp}$) calculated from the equation:

$$\eta_{sp} = \frac{T_1 - T_2}{T_2}$$

During both determinations the viscometer was situated in an accurately thermostated bath. The molecular weight of the methylated xylan was calculated from the equation:

$$M = \frac{C \times \eta_{sp}}{C \times Km}$$

where $C$ = Concentration in g. mols. of repeating unit per litre.

$Km$ = Staudinger's constant (155) taken as $12 \times 10^{-4}$.

$M$ = Molecular weight of methylated xylan.

The pertinent details of a series of viscosity determinations are given in the table on the next page. A graph of the specific viscosity plotted against the concentration of the m-cresol solution is given later (p. 113).
METHYLATED XYLAN

(See Fig. 2. p. 113.)

<table>
<thead>
<tr>
<th>Concentration in g./residues of C_5H_10O_4 per litre</th>
<th>Av. time of flow: Solution</th>
<th>No. of runs</th>
<th>Av. time of flow: Solvent</th>
<th>No. of runs</th>
<th>Bath temp. °C</th>
<th>¹sp/C</th>
<th>M</th>
<th>D.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0232</td>
<td>453.0</td>
<td>5</td>
<td>350.0</td>
<td>5</td>
<td>18.3</td>
<td>12.67</td>
<td>10,550</td>
<td>66</td>
</tr>
<tr>
<td>0.0150</td>
<td>397.2</td>
<td>5</td>
<td>331.8</td>
<td>5</td>
<td>17.3</td>
<td>12.80</td>
<td>10,670</td>
<td>67</td>
</tr>
<tr>
<td>0.0060</td>
<td>363.0</td>
<td>3</td>
<td>337.0</td>
<td>4</td>
<td>16.8</td>
<td>12.83</td>
<td>10,690</td>
<td>67</td>
</tr>
<tr>
<td>0.0030</td>
<td>246.0</td>
<td>4</td>
<td>237.0</td>
<td>4</td>
<td>22.5</td>
<td>12.67</td>
<td>10,550</td>
<td>66</td>
</tr>
</tbody>
</table>
Hydrolysis of the methylated xylan.

The methylated xylan (5.495 g.) and methanolic hydrogen chloride (600 ml.; 1%) were placed in a flask and the mixture heated, under reflux, on a water-bath. After 30 hours the solution remained slightly turbid and it was impossible to measure its optical rotation. The suspended material was removed by centrifugation (A) and was again heated, under reflux, with methanolic hydrogen chloride (150 ml., 1% for 2 h.). There was no apparent decrease in turbidity and heating was discontinued and the suspended material was centrifuged down (B). The residue (B) was heated in a sealed tube with sulphuric acid (0.5 ml., 1N; 9 h., 100°C). Chromatographic examination of the neutralised contents of the tube indicated that there were no sugars present.
Centrifugate A was neutralised by the addition of dry distilled diazomethane in ether. Centrifugate B was neutralised by the addition of silver carbonate and the excess of silver ions precipitated out as the sulphide by bubbling hydrogen sulphide through the solution; the mixture was filtered through a bed of filter-cel and charcoal. Centrifugates A and B were combined and the solvent evaporated at 30° under reduced pressure; a golden syrup was obtained.

The syrup was hydrolysed by heating it, under reflux, with hydrochloric acid (300 ml., 0.5N) on a boiling water-bath. Samples were withdrawn from time to time and their optical rotation (see below) was determined and when after 15 hours this was constant, the solution was cooled and neutralised by the addition of silver carbonate; traces of silver were removed as before. Removal of the solvent by distillation under reduced pressure at 35° yielded a golden-yellow syrup (5.997 g.; 98.5% of theoretical).

<table>
<thead>
<tr>
<th>Hours from start of hydrolysis</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>11</th>
<th>13</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a]_D^20</td>
<td>40.5</td>
<td>37.3</td>
<td>34.3</td>
<td>29.5</td>
<td>27.8</td>
<td>25.7</td>
<td>25.0</td>
<td>24.5</td>
<td>24.5</td>
</tr>
</tbody>
</table>
Separation of the hydrolysate of methylated xylan on a cellulose column.

Preparation of cellulose column.

A glass tube was packed with 80-mesh cellulose powder by the method recommended by Hough, Jones, and Wadman (156). The cellulose column (diameter 3.5 cm., length 88 cm.) was washed for 3 days with water, then with butanol (3 l.), and finally with 3 litres of light petroleum (b.p. 100-120°)—n-butanol (70:30), saturated with water (A).

Separation of the methylated xylan hydrolysate.

The major portion (5.930 g.) of the syrupy hydrolysate was dissolved, as far as possible, in butanol (2 ml.) and was pipetted on to the top of the cellulose column; the residual syrup was dissolved in butanol (2 x 1 ml.) and was also added to the column. The syrupy solutions were allowed to soak into the cellulose powder and a cotton-wool plug was then placed on top. Successive small additions (5, 10, 10 and 25 ml.) of Irrigant A were made to the top of the column each volume being allowed to soak in level with the top of the cellulose before the next addition was made. A constant-head reservoir containing Irrigant A was then placed in position on top of the column and the eluate collected in a series of tubes. The receiving tube (7-10 ml.) was changed automatically (156) every 8 or 12 minutes. When paper chromatographic examination of the eluate indicated that the dimethyl pentose fraction had been completely eluted,
the irritant was changed to butanol and then shortly afterwards to butanol half-saturated with water. It was not desirable to place this latter irritant directly on top of the butanol/light petroleum phase as this would have caused water droplets to be precipitated out from the latter irritant. When the monomethyl-pentose had been eluted the irritant was changed to water; the first part of the aqueous eluate was fractionally collected in tubes and the later part in a beaker.

A third of the content of every tenth tube was reduced to small volume and the concentrated solution was spotted on a chromatogram paper. Standard sugars were spotted on the paper and it was then irrigated (6 h) with Irrigant No. 5. Papers were treated with Spray No. 1 and spots were developed by heating which were correlated by position and colour to the standards: mono-0-methyl-xylose, 2:3-di-0-methyl-D-xylose, and tri-0-methyl-pentose. A spot attributed to an acid molecule was also noted. The contents of small numbers of tubes were combined after the sugars in them had been determined to be apparently the same and the solvents were removed by distillation under reduced pressure at 40-50°. Further examination of the fractions revealed no hitherto undetected sugars and the solutions were accordingly combined as indicated in the table.
### Fractions from Column

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Tube number</th>
<th>Sugar(s) in fraction</th>
<th>Weight of impure sugar(s) (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1-160</td>
<td>no sugar</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>161-190</td>
<td>trimethylpentoses</td>
<td>141.4</td>
</tr>
<tr>
<td>2</td>
<td>191-360</td>
<td>trimethylpentoses and dimethyl xylose</td>
<td>151.5</td>
</tr>
<tr>
<td>3</td>
<td>361-900</td>
<td>dimethylxylose</td>
<td>4907.0</td>
</tr>
<tr>
<td>-</td>
<td>900-1037)</td>
<td>no sugar</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1038-1080)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>1081-1160</td>
<td>monomethylpentose</td>
<td>132.2</td>
</tr>
<tr>
<td>5</td>
<td>1161-1501</td>
<td>no sugar</td>
<td>-</td>
</tr>
<tr>
<td>+ water wash</td>
<td>1502-1590</td>
<td>uronic acid</td>
<td>338.0</td>
</tr>
</tbody>
</table>

### Irrigant Changes on Column

<table>
<thead>
<tr>
<th></th>
<th>Number of collecting tube at time of change of Irrigant.</th>
<th>Number of tube in which Irrigant was first collected.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light petroleum / butanol 70:30 saturated with water</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Butanol</td>
<td>941</td>
<td>1038</td>
</tr>
<tr>
<td>Butanol (half-saturated with water)</td>
<td>961</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>1440</td>
<td>1516</td>
</tr>
</tbody>
</table>

Tube 1038, corresponding to the first tube of butanol, contained a small quantity of dimethyl-xylose (identified chromatographically).
The apparent recovery from the column = 95.6%. Correction for impurity in the fractions (p.100) reduces the above weight of material recovered to 5.259 g. (86.7% theoretical). About 1/30th. of the total amount of sugars eluted from the column was spotted on chromatogram papers. The recovery from the column corrected for this loss was ca. 92%.

It will be seen that in fraction 2 a mixture of di and trimethyl pentoses was present. Examination of fractions 1 and 2 in Irrigant No. 6 indicated the presence in both fractions of two sugars travelling at the same rate as 2:3:4-tri-0-methyl-D-xylose and 2:3:5-tri-0-methyl-L-arabinose (these sugars were not resolved in Irrigant No. 5). Fractions 1 and 2 were combined prior to rechromatographing. The combined fraction (292.9 mg.) was clouded with a wax-like impurity which was removed by filtration of an aqueous solution through 'filter-cel'. The purified syrup had \([\alpha]_D^{18} + 1.5^\circ\) (in water).

A sample (144.9 mg.) of the combined fractions (1 and 2) was spotted on a number of chromatogram papers (Whatman 3MM) and they were then irrigated (16-20 h) with Irrigant No. 6. Partial separation of the trimethyl-pentoses from one another and complete separation from the dimethyl-xylose was obtained. Side-strips of the papers were sprayed with aqueous aniline oxalate and it was observed that, during heating to develop spots, the spot produced by the trimethyl-arabinose appeared much more slowly than that.
produced by the trimethyl-xylose; in spite of some overlap it was therefore possible to accurately determine the areas of paper containing the different sugars.

On one paper complete separation of all three sugars had been obtained and it was retained for quantitative work. The positions of the sugars on the unsprayed areas of the other papers having been determined, four strips were cut from each paper each containing one of the following fractions a dimethyl-xylose, b trimethyl-xylose, c trimethyl-arabinose, d trimethyl-arabinose and trimethyl-xylose. The sugars were eluted with cold water (p. 52) and wax-like material was removed from the eluates by filtering it off. The fractions were taken to dryness.

**Estimation of the weights of individual sugars present in the fractions 1 and 2.**

The chromatogram on which complete separation of the three sugars had been obtained carried ca. 20 mg. of the combined fractions 1 and 2. Equal areas of paper, cut so as to contain only one sugar, were extracted with hot water by the method of Flood, Hirst, and Jones (139) and the aqueous extracts (5 ml.) were each diluted to 25 ml. The weights of sugar present in the extracts were determined by use of sodium hypoiodite (p. 54). The ratios of the sugars present were:

\[
\text{trimethyl-arabinose} : \text{trimethyl-xylose} : \text{dimethyl-xylose} = 0.78 : 1 : 0.47
\]
Hypiodite oxidation of the combined fraction (1 and 2) indicated a purity of 95.3%. The weight of the fraction corrected for impurity was therefore 278 mg. The weights of sugars present in the fraction were calculated to be:

- trimethyl-arabinose 77 mg.
- trimethyl-xylose 99 mg.
- dimethyl-xylose 46 mg.

Examination and identification of methylated sugars.

Fractions 1 and 2: examinations of sub-fractions b and c.

Sub-fraction b.

The syrup (42 mg.) had not crystallised after one week at 0°. Nucleation with a crystal of 2:3:4-tri-O-methyl-D-xylose led to the syrup's slow and complete crystallisation. The crystals had m.p. 89°. Recrystallisation from ether yielded crystals of m.p. 90-91°, not depressed on admixture with 2:3:4-tri-O-methyl-D-xylose. The crystals had $\left[a\right]_D^{18} + 20^°$ (c, 0.75 in water). X-ray powder diffraction photographs (irradiation 30 mins.) of the crystals and of 2:3:4-tri-O-methyl-D-xylose were indistinguishable.

A sample (ca. 2-3 mg.) of the fraction was heated (6 h; 95°) in a sealed tube with hydrochloric acid (1 ml.; W). Chromatographic examination of the neutralised solution on heavily spotted papers showed no trace of dimethyl xylose; it
was therefore assumed that no methyl di-\(\beta\)-methyl-D-xyloside was present in the fraction.

A sample (ca. 3 mg.) was demethylated by heating (4 min; 100°) in a sealed tube with hydrobromic acid (1 ml.; 48% w/w). The contents were rapidly cooled then neutralised. Chromatographic examination of the reaction mixture indicated the presence in it of xylose, monomethyl-xylose, and dimethyl-xylose: no arabinose was detected and it was therefore assumed that complete separation of the trimethyl-xylose from trimethyl arabinose had been obtained.

Preparation of 2:3:4-tri-\(\beta\)-methyl-N-phenyl-D-xylosylamine.

A sample (20 mg.) of sub-fraction b was heated under reflux for 6 hours with dry ethanolic aniline (1.5%; 1 ml.). The ethanol and the excess of aniline were removed by standing over paraffin wax and calcium chloride under reduced pressure in a vacuum desiccator. Nucleation of the resultant syrup with a minute crystal of 2:3:4-tri-\(\beta\)-methyl-N-phenyl-D-xylosylamine led to the syrup's slow and complete crystallisation (3 weeks at room temperature). During the crystallisation the product darkened. The crystals were contaminated with a little syrup but, due to their small amount, it was thought inadvisable to 'tile' them on porcelain. They were placed on filter-paper, under suction, and washed rapidly with ethyl acetate and with a little ether; fine colourless needles were obtained of m.p. 95°, not depressed.
on admixture with 2:3:4-tri-O-methyl-\(\text{H}\)-phenyl-\(\text{D}\)-xylosylamine. They had \([\alpha]_D^{18} +40^\circ\) (c, 0.1 in ethanol).

**Fraction c.**

The syrup (45 mg.) did not crystallise; chromatographic examination indicated that it was contaminated by a little trimethyl-xylose. A sample (3-4 mg.) was demethylated with hydrobromic acid as on the previous page. The reaction mixture was examined chromatographically and the presence of a trace of xylose confirmed. The fraction (42 mg.) was rechromatographed on thick paper (p.32) irrigated with Irrigant No. 6. The position of sugars was determined by spraying and developing the side-strips of the papers and appropriate areas were cut from the central unsprayed part of the paper so as to contain only one sugar. Elution of the areas of paper with cold water was followed by the removal of the solvent under reduced pressure at 40\(^\circ\): two fractions were obtained \[c\] trimethyl-arabinose (36 mg.), and \[c\] 2:3:4-tri-\(\text{O}\)-methyl-\(\text{D}\)-xylose (ca. 2 mg.). The syrup \[c\] had \([\alpha]_D^{19} +34.5^\circ\) (c, 0.6 in water).

**Preparation of 2:3:5-tri-\(\text{O}\)-methyl-L-arabonamide.**

A sample (28 mg.) of syrup \[c\] was dissolved in water (1 ml.) and liquid bromine (0.2 ml.) added; the solution was left at room temperature for three days with frequent shaking. The bromine was removed by aeration of the solution, and the product was neutralised by the addition of a little silver
carbonate; traces of silver were removed in the usual way (p.78). The solvent was removed by distillation under reduced pressure at 40°; a pale-brown, highly-viscous syrup remained. The syrup was distilled, under reduced pressure, in a micro-distillation apparatus and the distillate collected on a cold-finger. The pale-brown lactone which was obtained was treated with saturated anhydrous methanolic ammonia (1 ml.) for 18 hours at 0°. The solvent was removed by distillation under reduced pressure at 35° and a pale straw-coloured syrup was obtained. Nucleation with an authentic crystal of 2:3:5-tri-O-methyl-L-arabonamide led to the syrup's crystallisation within a few days. The crystals on recrystallisation from ethyl acetate had \([\alpha]_D^{20} -16.0°\) (c, 1.0 in water), and m.p. 136-137°, not depressed on admixture with 2:3:5-tri-O-methyl-L-arabonamide. X-ray powder diffraction photographs (irradiation for 40 min.) of the authentic trimethyl-arabonamide and of the amide formed from fraction c1 indicated that the two lots of crystals were identical.

**Fraction 3.**

The syrup (4.907 g.) was clouded with a wax-like material which was removed on filtration of an aqueous suspension of the material through a little charcoal and filter-cel. The water was removed by distillation under reduced pressure at 45° and left a pale-yellow, viscous syrup. It travelled on paper chromatograms irrigated with Irrigants Nos. 1, 4, and
5 at the same rate as 2:3-di-O-methyl-D-xylose. Buffered hypoiodite oxidation (p.54) indicated that the syrup was 94% pure (Found: OMe, 34.4%. Calculated for C7H14O5: 34.8%).

A sample (20 mg.) was demethylated by heating (5 min.; 100°) with hydrobromic acid (1.5 ml.; 48% w/w). The reaction mixture was neutralised and then examined chromatographically: sugars travelling at the same rate as xylose, monomethyl-xylose and 2:3-di-O-methyl-D-xylose were detected; there was no trace of arabinose.

After over a year in a refrigerator at 0 ° the syrup had not started to crystallise. It was divided into two equal portions and to one there was added a small crystal of 2:3-di-O-methyl-a-D-xylose and to the other a crystal of the corresponding β anomer. In both cases crystallisation started rapidly; it was complete within three days in the case of the portion seeded a; it took a fortnight in the case of the sample seeded β. The two crystalline masses were separately ground down and then washed rapidly with acetone (6 x 5 ml.) then with ether (3 x 5 ml.) and the wash liquors were filtered off under suction.

The anomers used for nucleation are called a and β. The crystals obtained on seeding with a and β are distinguished as a' and β' respectively. The melting points and mixed melting points were as follows:
Seed crystals: α 79-80°, β 73-5-78°.
Unknown forms: α' 78-81°, β' 82-84°.
Mixtures: α' + β' 78-5-83.5°, β' + α 64-84°, α' + α 65-81°.

From a consideration of the mixed melting points it will be obvious that the samples both crystallised in the β form. This is confirmed by the specific rotations of samples α' and β'.

**Rotation of sample seeded α.**

c, 0.64 in water.

<table>
<thead>
<tr>
<th>Minutes after solution</th>
<th>13</th>
<th>15</th>
<th>16</th>
<th>20</th>
<th>25</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>[α]$_D$</td>
<td>-20.3°</td>
<td>-12.5°</td>
<td>-9.38°</td>
<td>-5.45°</td>
<td>+3.12°</td>
<td>+10.16°</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minutes after solution</th>
<th>70</th>
<th>105</th>
<th>145</th>
<th>240</th>
<th>330</th>
</tr>
</thead>
<tbody>
<tr>
<td>[α]$_D$</td>
<td>+21.8°</td>
<td>+25.8°</td>
<td>+27.3°</td>
<td>+27.8°</td>
<td>+27.3°</td>
</tr>
</tbody>
</table>
Rotation of sample seeded $\beta$.

$c, 0.7$ in water.

<table>
<thead>
<tr>
<th>Minutes after solution</th>
<th>12</th>
<th>35</th>
<th>90</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\alpha]_D^{13}$</td>
<td>-18.1°</td>
<td>+7.5°</td>
<td>+23.0°</td>
<td>+26.2°</td>
<td>+26.2°</td>
</tr>
</tbody>
</table>

Preparation of 2:3-di-$\alpha$-methyl-$\alpha$-phenyl-$\alpha$-xylosylamine.

A sample (97 mg.) of fraction 3 was added to a molar proportion of freshly-distilled aniline in dry ethanol (5 ml.). The solution was boiled under reflux on a water-bath for three hours then the solvent was removed under reduced pressure in a vacuum-desiccator over paraffin wax and phosphorous pentoxide. Crystallisation took four weeks to complete and then yielded colourless crystals which were slightly contaminated with syrup. After tiling them on porcelain, the crystals were recrystallised from ethyl acetate. They had m.p. 144-145°, undepressed by admixture with 2:3-di-$\alpha$-methyl-$\alpha$-phenyl-$\alpha$-xylosylamine. X-ray powder diffraction photographs (irradiation 30 min) were taken of the above anilide and of those derived from 2:3, 2:4, and 3:4-di-$\alpha$-methyl-$\alpha$-xyloses. The photograph produced by the anilide of the fraction 3 corresponded to that of 2:3-di-$\alpha$-methyl-$\alpha$-phenyl-$\alpha$-xylosylamine; it was distinctly different from those of the other two anilides.
Preparation of 2:3-di-O-methyl-D-xylonamid.

A sample (110 mg.) of fraction 3 was dissolved in water (2.5 ml.) and liquid bromine (0.5 ml.) added, and the solution frequently shaken over a period of six days. The excess of bromine was removed by aeration of the solution, which was then neutralised by the addition of a little silver carbonate: traces of silver were removed in the usual way. The solvent was removed by distillation under reduced pressure at 35° and the product was heated (3 h; 90°/25mm) to convert the acid formed to the lactone.

After treatment of the lactone for forty hours with an excess of saturated, anhydrous methanolic ammonia the solvent was removed at 30° under reduced pressure. The brownish non-crystalline product which was obtained crystallised from solution in acetone on the addition of a crystal of 2:3-di-O-methyl-D-xylonamide. The prepared amide had m.p. 136.5°, not depressed by admixture with 2:3-di-O-methyl-D-xylonamide.

Fraction 4.

Fraction 4 was a straw-coloured syrup (132.2 mg.). Its rate of movement on chromatograms irrigated with Irrigants Nos. 2, 4 and 5 was the same as those of 2 and 3-O-methyl-D-xyloses. It was noted that the colour reactions of the two mono-O-methyl-D-xyloses were different; on papers sprayed with p-anisidine hydrochloride
(in butanol) the 2-α-methyl-D-xylose gave rise, on heating, to a purplish-brown spot, while the 3-α-methyl-D-xylose gave a yellowish-brown spot. Another colour difference will be mentioned shortly. By means of this colour reaction the sugar was tentatively identified as 2-α-methyl-D-xylose.

The syrup which was highly viscous had completely crystallised within a fortnight to give colourless needles. A specimen was washed rapidly with analar acetone; the residual crystals had m.p. 132-133° alone or admixed with 2-α-methyl-D-xylose. Hypoiodite oxidation indicated that the fraction was 93% pure. It had [α]_D^23 + 34.0° (c, 1 in water) (Found: OMe, 18.3%. Calculated for C_6H_{12}O_5 18.9%). The X-ray powder diffraction photograph of crystals of fraction 4 was compared with those produced by 2 and 3-γ-methyl-D-xyloses and it was found to correspond to the former.

Ionophoretic examination of the fraction on papers wet with borax solution (0.05M) confirmed the above characterisation. Details are given later (p. 96).

Preparation of 2-γ-methyl-N-phenyl-D-xylosylamine.

The anilide was prepared from a sample (25 mg.) of the fraction in the usual way (p. 91). Crystallisation was slow but complete; it did not require the addition of a seed crystal for initiation. The crystals had m.p. 123.5°.
not depressed on admixture with 2-\(\text{\text{\text{-}}}m\)-methyl-\(\text{\text{\text{-}}}m\)-phenyl-\(\text{\text{\text{-}}}d\)-xylosylamine.

**Fraction 5.**

The syrup (336 mg.) was dark-brown and obviously impure; it was incompletely soluble in a large volume of methanol. A sample (ca. 5 mg.) of the residue, which was insoluble in water and in ethyl acetate, was heated (6 h; 100°) in a sealed tube with sulphuric acid (2 ml.; 2N). The mixture, after neutralisation, was examined chromatographically; no reducing sugars were detected and the residue was therefore discarded. The brown methanolic centrifugate was decolourised by boiling it with a little charcoal for a few minutes. The charcoal was filtered off and the methanol was removed by distillation under reduced pressure at 35°; a pale straw-coloured syrup (266 mg.) remained. It had \([c]_D^{10} +54^\circ\) (c, 0.5 in water). The syrup was examined on a chromatogram paper irrigated for 36 hours by Irrigant No. 2. After spraying with Spray No. 1 the paper was heated and a single spot (Rg, 0.12) developed; it was red in daylight and orange in ultraviolet light.

A sample (135.9 mg.) of the fraction was boiled for 6 hours under reflux with dry methanolic hydrogen chloride (50 ml.; 1.5%). The solution was then neutralised with silver carbonate and the silver residues filtered off; remaining silver was removed in the usual way (p. 78).
The methanol was evaporated under reduced pressure at 30° and the residue was taken up in dry ether (25 ml.). The ether solution was added dropwise, over a period of three hours, to a suspension of lithium aluminium hydride (250 mg.) in dry ether (25 ml.) boiling under reflux. Throughout the reaction the mixture was vigorously stirred. After 5 hours, unreacted hydride was decomposed by the addition of a little water and the emulsion formed was made acid to methyl orange by the addition of sulphuric acid (2N). The ether was removed under reduced pressure at room temperature, and the aqueous solution was extracted with chloroform (6 x 50 ml.). The chloroform on evaporation left a syrup (77.8 mg.).

The syrup was hydrolysed with hydrochloric acid (30 ml., 0.5N; 8 h, 100°). The reaction-mixture was neutralised with silver carbonate and the silver residues and soluble silver removed in the usual way. The water on removal by distillation under reduced pressure at 50° left a syrup (60.0 mg.). This syrup was examined chromatographically on papers on which the following standard sugars were also spotted: 2:3:4-tri-0-methyl-D-glucose, 2-0-methyl-D-xylose and 3-0-methyl-D-xylose. Papers were irrigated with Irrigants Nos. 4 and 6 and were then developed after being sprayed with Spray No. 1. Two spots were observed: one corresponded in position and colour to 2:3:4-tri-0-methyl-D-glucose; the other from its position could be ascribed either to 2 or to 3-0-methyl-D-xylose.
The colour of the spot produced by the mono-\(\beta\)-methyl-D-xylose indicated strongly that it was produced by the 3 methyl derivative. Aniline oxalate solution gave the following colours with these two sugars:

- **2-\(\alpha\)-methyl-D-xylose**: in daylight and in ultra violet light - red-brown.
- **3-\(\alpha\)-methyl-D-xylose**: in daylight - burgundy; in ultra-violet light - practically black with purple edges.

Chromatography, in the above solvents at any rate, is of little use in achieving a separation of the monomethyl-xyloses: recourse was therefore made to ionophoresis.

**Ionophoresis of the product obtained on reduction and hydrolysis of fraction E.**

Full details of the procedure are given earlier (p. 53). On to a paper, soaked in 0.05M borax solution, there were spotted: the hydrolysate and, as standards, 2:3:4-tri-\(\alpha\)-methyl-D-glucose and 2 and 3-\(\alpha\)-methyl-D-xyloses. A potential difference of 450 volts was applied between the ends of the paper for 4 hours; during this time the current flowing through the paper rose from 10 to 20 mA. On Paper 2 in the accompanying photograph (p. 97) it will be seen that the hydrolysate gave rise to two spots which, by colour and position, could be correlated to 2:3:4-tri-\(\alpha\)-methyl-D-glucose and to 3-\(\alpha\)-methyl-D-xylose.

**Fraction 4 (p. 92):** The monomethyl-xylose fraction from the column was also examined by ionophoresis (Paper 1). It will be seen that this paper confirms that the sugar was 2-\(\alpha\)-methyl-D-xylose.
PAPER IONOPHORETGRAMS

PAPER 1

[4 h at P.D. of 450 v. Current 10-15 mA].

(a) 3-O-methyl-D-xylose.
(b) Fraction 4.
(c) 2-O-methyl-D-xylose.

(d) 2:3:4-tri-O-methyl-D-glucose.
(e) Reduced and hydrolysed Fraction 5.
(f) 2-O-methyl-D-xylose.
(g) 3-O-methyl-D-xylose.

Both papers were sprayed with Spray No. 1 to which there had been added a few drops of glacial acetic acid. The papers were developed by heating at 110°.
The above separations were repeated on other ionophoretogram papers and the results were confirmed.

It should be noted that the monomethyl-xyloses from fraction 4, and from the hydrolysate of fraction 5, were different and that, moreover, only one monomethyl-xylose was present in each fraction.

**Separation of monomethyl-xylose from trimethyl-hexose.**

The reduced and hydrolysed fraction 5 was spotted on chromatogram papers (Whatman 3MM) which were then irrigated for 15-18 hours with Irrigant No. 6. The position of the monomethyl-xylose, and of the trimethyl-hexose were determined by the usual method of spraying side-strips. The central, unsprayed, areas of the papers which were thus determined to contain sugars were eluted, separately, with cold water and the eluates evaporated to dryness under reduced pressure. Two subfractions were obtained.

**The trimethyl-hexose sub-fraction.**

The syrup (21 mg.) was boiled for 6 hours under reflux with dry methanolic hydrogen chloride (3 ml.: 2%); it then did not reduce Fehling's solution. Neutralisation with silver carbonate was followed by the removal of the excess of silver ions in the usual way. After filtration of the solution through a bed of filter cel and a little charcoal the methanol was distilled off at 35° under reduced pressure.
The colourless film of syrup which was obtained was seeded with methyl 2:3:4-tri-3-methyl-β-D-glucoside and after three days at 0° over phosphorous pentoxide the syrup appeared to have crystallised completely. The fine crystals were washed rapidly with ice-cold ether and dried. They had m.p. 89.5°, not depressed on admixture with methyl 2:3:4-tri-3-methyl-β-D-glucoside.

Other work on this fraction has already been described in an earlier section on ionophoresis (p.96).

The monomethyl-xylose sub-fraction.

A colourless syrup (28 mg.) had \([\alpha]_{D}^{13} + 19.5°\) (c. 0.3 in water). A sample (7.2 mg.) of the monomethyl-xylose was boiled under reflux with ethanolic aniline (1 ml., 1%). Difficulty had been experienced in earlier attempts to prepare the anilide of this fraction due to darkening of the reaction mixture. To prevent oxidation and possible photodecomposition of the aniline, a stream of nitrogen was passed through the flask and it was kept in the dark. The ethanol and remaining aniline were removed under reduced pressure over calcium chloride and paraffin wax in a vacuum-desiccator. The anilide crystallised after two weeks during which time it was kept under nitrogen and at 0°. Tiling of the product was impracticable due to the small amount of material. The crystals were freed from syrup by placing them on a filter-
paper under suction and washing them rapidly with ether and then with a little ethyl acetate. The crystals had m.p. 136-137°; there was no authentic sample of 3-0-methyl-N-phenyl-D-xylosylamine with which to do a mixed melting-point.

Other work on this fraction has been mentioned in the section on ionophoresis (p. 96).

**Summary of the weights of methylated sugars separated on the cellulose column.**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Weight of impure sugar recovered from column. (mg.)</th>
<th>Fraction Number</th>
<th>Purity by hypiodite</th>
<th>Corrected weight (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-0-methyl-D-xylose</td>
<td>132.2</td>
<td>4</td>
<td>93%</td>
<td>123</td>
</tr>
<tr>
<td>2,3-di-0-methyl-D-xylose</td>
<td>4,907.0</td>
<td>3 and part 2</td>
<td>94%</td>
<td>4,620</td>
</tr>
<tr>
<td>2,3,4-tri-0-methyl-D-xylose</td>
<td>127</td>
<td>1 and 2</td>
<td>95.3%</td>
<td>99</td>
</tr>
<tr>
<td>2,3,5-tri-0-methyl-L-arabinose</td>
<td>98</td>
<td>1 and 2</td>
<td>95.3%</td>
<td>77</td>
</tr>
<tr>
<td>3-0-methyl-2-0-(2,3,5-tri-0-methyl-D-glucuromosyl)-D-xylose</td>
<td>338.0</td>
<td>5</td>
<td>-</td>
<td>293</td>
</tr>
</tbody>
</table>

Total 5,529
Examination of an acidic fraction obtained from the hydrolysate of oat straw xylan.

Xylan (14 g.) was heated with sulphuric acid (100 ml.; 0.25N) for 4 hours at 100° and the normality of the acid was then increased to 0.5 by the addition of 2N sulphuric acid and heating was continued for a further 4 hours. A brown residue was centrifuged down and the centrifugate was neutralised by the addition of barium carbonate. The mixture was filtered then the filtrate was de-cationised by passing it through a column (30 x 3 cm.) of Amberlite resin IR 120(H). The resin was washed with water (500 ml.) and the eluate passed through a column (26 x 3 cm.) of Amberlite resin IR 4B(OH) to adsorb the acid-fraction of the hydrolysate. The column was washed with water (ca. 20 l.) until long after the eluate did not reduce Fehling’s solution. The resin was treated with sulphuric acid (2N; 1,500 ml.) to desorb the acid-fraction and the acidic solution was neutralised with barium carbonate and barium hydroxide. The mixture was filtered and the filtrate freed from barium ions by passing the latter through a column (30 x 3 cm.) of Amberlite resin IR 120 (H). The solvent was removed by distillation under reduced pressure at 50° and the residue was examined chromatographically; it was found to contain a high proportion of xylose. The residue was dissolved in water and was re-adsorbed on a column (20 x 3 cm.) of Amberlite resin IR 4B(OH) which was washed with water
(10 l.). The earlier desorption and neutralisation procedures were repeated and the solvent was partially removed from the eventual aqueous solution by distillation under reduced pressure at 50°; remaining water was removed by freeze-drying. The 'felt' (200 mg.) obtained was examined chromatographically on papers irrigated with Irrigants Nos. 2 (acid) and 4 (non-acid). Three spots were developed on both papers after they had been treated with Spray No. 1. One spot was attributable to xylose and one to material which had not moved from the starting-line on either paper. The third spot had an Rf value ten times as great in the acid as in the non-acid solvent; it was assumed to be due to an acidic molecule. It was not possible to say, from visual inspection of the spots, what proportion of the material spotted on the papers was represented by the third spot.

A potentiometric titration of a sample (13.40 mg.) of the 'acid-fraction' with carbonate-free sodium hydroxide (0.01946 N) indicated that the material had an equivalent weight of 1,313. If it is assumed that the acid-fraction was present as the monomethyl-aldobiuronic acid (equivalent weight, 340) then it would appear that the fraction was only 26% pure.

Reduction of the 'acid-fraction'.

An unsuccessful attempt was made to reduce a sample (16 mg.) of the acid-fraction, suspended in ether, by treatment with lithium aluminium hydride.
A sample (25 mg.) of the acid-fraction was heated under reflux for 6 hours with dry methanolic hydrogen chloride (10 ml.; 1.5%). The solution was neutralised with silver carbonate and the residues and the remaining traces of silver were removed in the usual way. The methanol was removed by distillation under reduced pressure and the residue was taken up in dry ether (20 ml.). The ether solution was heated under reflux and to it there were added portions (ca. 10 mg.) of lithium aluminium hydride (100 mg.) over a period of 6 hours. The mixture was cooled and unreacted hydride was destroyed by the addition of a little water. The ether was removed by distillation and the residue taken to pH 7 with sulphuric acid (2N). The solution was de-ionised by successive treatments with Amberlite resins IR 120(H) and IR 4B(OH) and the volume of the aqueous solution was then reduced to 5 ml. and sulphuric acid (5 ml.; 2N) was added. The resultant solution was heated in a sealed tube (100°; 6 h). The product was cooled and, after being neutralised with barium carbonate, was examined chromatographically (Irrigants Nos. 1 and 4). Papers were sprayed (Spray No. 1) and then developed by heating; identical spots were observed on each. One spot had travelled at the same rate as xylose and one at the rate of 4-O-methyl-D-glucose; the latter was much fainter than the former. There was possibly an indication of a trace of glucose.
Milled oat straw was delignified by treating it with acid chlorite, the solution being buffered to pH 5-6 to minimise the danger of any acidic hydrolysis of the hemicelluloses. The resultant holocellulose was treated with dilute alkali at room temperature in the absence of oxygen and the hemicelluloses were precipitated from the acidified extract by the addition of acetone. A hydrolysate of the hemicelluloses was shown, on chromatographic examination, to contain arabinose and xylose in the ratio of 1 to 17 and also traces of glucose and of galactose. The hemicellulosic material had $\left[a\right]_{D}^{18} -94.7^\circ$ (c, 0.6 in N NaOH). It was treated with Fehling's solution and the precipitated copper complex which formed was separated and then decomposed by treatment with acid. During this and two subsequent complex formations and decompositions the ratio of arabinose to xylose fell to 1 to 33. Further reprecipitations of the xylan as its copper complex failed to reduce the percentage of arabinose residues in the regenerated xylan and this xylan was used in all subsequent work. It had $\left[a\right]_{D}^{19} -95.0^\circ$ (c, 0.5 in N NaOH); lignin content of 3.3%; ash of 0.8%; uronic anhydride content of 3.5%; and a methoxyl content of 0.5%.

The xylan was methylated by two successive treatments with methyl sulphate and aqueous sodium hydroxide (40%) in an atmosphere of nitrogen. The product was further
methylated by treating it with neutral methyl iodide and silver oxide giving a derivative which, on fractional dissolution in mixtures of light petroleum (b.p. 65-70°) and chloroform, gave six fractions ranging in methoxyl content from 36.8-38.2% (Calculated for C7H14O4, 38.75%). The fraction with the methoxyl content of 38.2% was used in all subsequent work on the methylated xylan. It had [α]D17 +37.0° (c, 0.91 in CHCl3). It was hydrolysed by successive treatment with methanolic hydrogen chloride (1%) and hydrochloric acid (0.5N) until the rotation of the reaction mixture was constant. The mixture of reducing sugars obtained had [α]D20 +24.5° (c, 2.0 in 0.5 N HCl). The syrup, after being fractionated by eluting it from a column of powdered cellulose, was recovered in 92% yield. Five fractions were obtained: Fractions 1 and 2 contained mixtures of sugars while Fractions 3-5 each contained only one sugar. The mixed fractions (1 and 2) were combined and were then separated on a number of thick paper chromatograms irrigated with benzene/ethanol/water (167:47:15 - top layer) to yield four sub-fractions: a, identical to fraction 3; b and c each containing only one sugar; and d containing a mixture of the sugars present in sub-fractions b and c. On one of the chromatogram papers complete separation of all of the sugars had been obtained (i.e. there was no sub-fraction d) and the relative proportions of the individual sugars which were eluted from it were determined by hypiodite oxidation (p.54) and
from these proportions the weights of individual sugars present in the combined fractions (1 and 2) were calculated.

The various sugars were characterised by the formation of crystalline derivatives and the melting points, specific rotations, and in some cases the X-ray powder diffraction photographs, of the sugars and derivatives were taken where possible.

Sub-fraction b (from the combined fractions 1 and 2) crystallised and was identified as 2:3:4-tri-O-methyl-D-xylose and this identification was confirmed by the formation of the corresponding crystalline anilide. Sub-fraction c (from the combined fractions 1 and 2) was characterised as 2:3:5-tri-O-methyl-L-arabinose by the formation of the crystalline amide of the corresponding trimethyl-arabonic acid. Fraction 3 crystallised on nucleating it with either a crystal of the α or of the β anomer of 2:3-di-O-methyl-D-xylose; in both cases the crystalline product was identified as 2:3-di-O-methyl-β-D-xylose. Confirmation of this characterisation came from the preparation of its anilide and of the crystalline amide of the corresponding dimethyl-xylonic acid. A sample of the dimethyl-xylose was demethylated by treating it with hydrobromic acid and the neutralised reaction mixture was examined chromatographally; there was no trace of any arabinose or of any of its methylated derivatives and it was therefore concluded that no dimethyl-arabinose was
present in the fraction; a possibility that had to be considered since dimethyl-xyloses and dimethyl-arabinoses are not readily chromatographically separable.

Fraction 4 crystallised and was identified as 2-0-methyl-D-xylose and this was confirmed by the preparation of the corresponding crystalline anilide. The monomethyl-xylose was found to travel on ionophoretograms at the same rate as 2-0-methyl-D-xylose and much slower than 3-0-methyl-D-xylose.

Fraction 5, an acid fraction, was treated with methanolic hydrogen chloride and the product was then reduced by treatment with an ethereal solution of lithium aluminium hydride. From the reaction-mixture a sugar was isolated which on hydrolysis gave two sugars which were, after chromatographic separation, identified as 3-0-methyl-D-xylose and 2:3:4-tri-0-methyl-D-glucose by the formation of the anilide of the former and the 0-methyl glucoside of the latter. Support for these identifications came from the ionophoretic examination of the two sugars. The intensity of the spots produced by the sugars on sprayed and developed chromatograms and ionophoretograms indicated that the two sugars were present in approximately equal amounts.

The weights of the various sugars recovered from the column of powdered cellulose were corrected for impurities.
and from those corrected weights the molar ratios of the various sugars present in the hydrolysate of the methylated xylan were calculated to be as follows:

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-O-methyl-D-xylose</td>
<td>1.14</td>
</tr>
<tr>
<td>2:3-di-O-methyl-D-xylose</td>
<td>40.0</td>
</tr>
<tr>
<td>2:3:4-tri-O-methyl-D-xylose</td>
<td>1.00</td>
</tr>
<tr>
<td>2:3:5-tri-O-methyl-L-arabinose</td>
<td>0.79</td>
</tr>
<tr>
<td>tetra-O-methyl-aldobiuronic acid</td>
<td>1.06</td>
</tr>
</tbody>
</table>

An acidic sugar was isolated from a hydrolysate of the xylan and, after it had been treated with methanolic hydrogen chloride to form the methyl ester and glycoside, the product was reduced with lithium aluminium hydride (163) in ether. The reduced product was hydrolysed with sulphuric acid and the neutralised hydrolysate was examined chromatographically; it contained two sugars which travelled at the same rates as xylose and 4-O-methyl-D-glucose and there was possibly a trace of glucose also present.

The results of the copper complex fractionation of the hemicelluloses indicated that the original crude material contained a mixture of hemicelluloses [a hypothesis that gained support in later work (p.119)], whilst the constant composition of the xylan regenerated after the later copper complex fractionations indicated that the arabinose and the xylose residues were present in the same molecular species. The low but definite methoxyl content of the xylan suggested, by analogy with many other xylans, that the uronic acid
residues present in the molecule might be partly methylated.

The absence of any indication of dimethyl-arabinose in the hydrolysate of the methylated xylan and the presence in it of trimethyl-arabinose indicated that all of the arabinose residues in the xylan were present as end-groups and that none were present in the xylan linked through two positions. The absence of dimethyl-arabinose also proved that no araban was present as contaminant of the xylan.

It is evident that in the tetra-O-methyl-aldobiuronic acid the C(1) position of the 2:3:4-tri-O-methyl-D-glucuronosyl group could be glycosidically attached to either the C(2) or the C(4) position of the 3-O-methyl-D-xylose residue. The former linkage appears to be the more likely unless it is assumed that this xylose residue is unique in the xylan molecule in being bound to another xylose residue by a 1→2 instead of by the usual 1→4 glycosidic link between such residues. A survey of other xylan molecules investigated shows that the linkage between the two residues in the various aldobiuronic anhydrides is normally believed to be either 1→2 or 1→3. The high specific rotation of the acid fraction from the hydrolysate of the methylated xylan indicated that the two residues were probably linked by an α bond.

Reduction and hydrolysis of an acid fraction obtained from the hydrolysate of xylan yielded 4-O-methyl-D-glucose and xylose; those sugars would be obtained on the reduction and
hydrolysis of 2-O-(4-O-methyl-a-D-glucuronosyl)-D-xylose

From the high negative rotation of both the xylan and the methylated xylan it is deduced that the xylose residues must be linked, principally at any rate, by \( \beta \) glycosidic bonds. If it is assumed that in the methylated xylan molecule there is only one non-reducing terminal xylose residue (an assumption that will be justified later) then one formula for the xylan molecule is shown below which would satisfactorily account for the experimental evidence. It is not possible by present day techniques to decide the actual or even the relative positions of the side-chains and a modification of formula I is possible in which the positions of the side-groups are reversed.

\[
\text{Xpl} \rightarrow (4\text{Xpl})_a \rightarrow 4\text{Xpl} \rightarrow (4\text{Xpl})_b \rightarrow 4\text{Xpl} \rightarrow (4\text{Xpl})_c \rightarrow 4\text{Xpl}
\]

\[
\text{II}
\]

\[
\begin{array}{cccc}
\text{I} & \text{II} \\
\text{Xpl} & (4\text{Xpl})_a & 4\text{Xpl} & (4\text{Xpl})_b & 4\text{Xpl} & (4\text{Xpl})_c & 4\text{Xpl} \\
\uparrow & \uparrow & \uparrow & \uparrow & \uparrow & \uparrow & \uparrow \\
1 & 1 & (Xp)_d & \text{mGA} & \text{Af} & \text{a} & \text{b} & \text{c} & \text{d} = 39 \\
\end{array}
\]

\[
\text{Af} = \text{arabofuranose residue} \\
\text{mGA} = 4-O-methyl-D-glucopyranuronic acid residue.
\]

\[
\text{a, b, c, or d may have any value from 0 to 39.}
\]

\[
\text{Xp} = \text{xylopyranose residue.}
\]
The methylated xylan which would theoretically be derived on complete methylation from a xylan of the above formulation would on hydrolysis give rise to equimolar amounts of 2-\(\varphi\)-methyl-\(D\)-xylose and of 2:3:5-tri-\(\varphi\)-methyl-\(L\)-arabinose. As will be seen from the data earlier (p.108) the molar amounts of the two sugars were not quite equal. It is of interest to note that several workers have found an excess of 2-\(\varphi\)-methyl-\(D\)-xylose over the theoretical amount which would be required by their suggested formulations for the methylated xylan. It is possible that the excess of 2-\(\varphi\)-methyl-\(D\)-xylose could be explained as being due either to under-methylation of the xylan or to partial demethylation of the methylated xylan or of its hydrolytic derivatives. It should however be noted that in the case of the oat straw methylated xylan hydrolysate there was no trace of any 3-\(\varphi\)-methyl-\(D\)-xylose detectable on ionophoretic examination of the monomethyl-xylose fraction.

If it may be assumed that the linkage of the arabinose residues in xylans from different sources is similar then, from Perlin's work on wheat flour (80), the favoured structure for the xylan would appear to be one in which the arabinose is attached directly to the main xylan chain as in formula II.
The specific viscosity of the methylated xylan in m-cresol was determined and from it, using the Staudinger constant applied to the corresponding cellulose derivative, the molecular weight of the methylated xylan was calculated to be approximately 10,600 and the degree of polymerisation to be approximately 66. These values are of the same order as those obtained by methylation and periodate oxidation studies.

Results of periodate oxidation studies are in good agreement with those that would be expected from the periodate oxidation of a xylan with structure I or II. After 432 hours the uptake of periodate was nearly constant and it was then found that 1.007 moles of periodate had been consumed for every C_5H_3O_4 unit; xylans with either of the above structures would be expected to consume 1 mole. The formic acid released on oxidation of the xylan with periodate ions did not reach a constant value (Fig. 3), but by extrapolating the curve to zero time (in order to correct for the effect of over-oxidation) it was determined that 1 mole
Concentration mg./litre.

VISCOsITY OF METHYLATED XYLan
PLOTTED AGAINST ITS CONCENTRATION
IN M-CRESOL.

FIG. 2.
PERIODATE OXIDATION

MOLES FORMIC ACID RELEASED PER \( \text{C}_3\text{H}_4\text{O}_4 \) RESIDUE \( \times 10^2 \)

PLOTTED AGAINST HOURS.

Fig. 3.
of formic acid was released for every 15.7 sugar residues (calculated as xylose residues). The reducing group would give rise to two molecules of formic acid and the non-reducing group to one molecule of the acid, but the glucuronic acid residue since it was methylated in the C(4) position would not be expected to release any acid nor would the arabofuranose side-group. If therefore the xylan had either of the above structures then, from a study of the formic acid released, it follows that the molecule contained 47 sugar residues; a value in good agreement with the results of methylation studies which indicated a degree of polymerisation of 45. If, on the other hand, the molecule possessed a structure of type III, that is one in which the molecule had two non-reducing xylose end-groups then the degree of polymerisation would, on the basis of the formic acid released, be 63. It therefore appears reasonably certain that the molecule did not have a structure of type III.

\[ Xpl \to (\text{Xpl})_a \to \frac{1}{2}Xpl \to (\text{Xpl})_b \to \frac{1}{3}Xpl \to (\text{Xpl})_c \to \frac{1}{3}Xpl \to (\text{Xpl})_d \to \frac{1}{4}Xpl \to \text{Xp} \]

\[ a + b + c + d + e = 37 \]
Following hydrolysis of the periodate-oxidised xylan the hydrolysate was examined chromatographically and was found to contain a trace of xylose which was assumed to have arisen from the branch points present in the molecule since xylose residues at such points would not be attacked by periodate.

It is concluded that the xylan molecule consisted of a chain of approximately 40 β-D-xylopyranoside residues linked 1→4 terminated by one reducing and one non-reducing xylose residue, and having attached as side-groups an arabofuranose residue linked either α or β 1→3 and a 4-O-methyl-D-glucopyranuronosyl residue linked α 1→2.

The above structure proposed for the oat straw xylan contains both arabinose and uronic acid residues and in this respect is similar to the structures ascribed, following methylation studies, to xylans isolated from wheat straws by Adams (p.26), Roudier (p.26), and by Meek (p.27), and to a xylan isolated from wheat leaf by Adams (p.30). Weihe and Phillips isolated a xylan from wheat straw (p.25) and one from corn stalks (167) and Phillips and Davis (168) isolated one from alfalfa hay; those three xylans contained both arabinose and uronic acid residues, but the structures were not determined. In all of the above xylans, excluding those extracted by Roudier
and by Weihe and Phillips from wheat straws, the xylans contained a higher proportion of uronic acid residues than of arabinose residues. The xylans isolated by Roudier and by Weihe and Phillips from wheat straw contained equimolar amounts of the two residues. The xylan isolated by Roudier was similar to the oat straw xylan in carrying only one of each residue and in this case, as in many others, the uronic acid residue carried a methoxyl group on C(4).

Other xylans investigated have been found to contain uronic acid residues but no arabinose residues. They include xylans from flax (p.36), pear cell-wall (p.39), beechwood (p.40), and birchwood (p.43). Certain xylans contain arabinose residues but no uronic acid residues, for example, a xylan from wheat flour (p.32), and one from esparto grass (p.34). With the exception of a xylan isolated from wheat bran (p.30) the arabinose appears to be present exclusively as side or end group.

One respect in which the oat straw xylan appears to differ from other straw xylans hitherto investigated is in the position of the attachment of the uronic acid residue to the main xylan chain. In the oat straw xylan it is attached to C(2) position of a xylose residue, whereas in all structural investigations of straw xylans so far published it has been concluded that the position of attachment was to C(3). A 1→2 link has, however, been found in the above-mentioned xylans from birch and beech woods.
**AN OAT STRAW**

**ARABINOSE-RICH HEMICELLULOSE**

<table>
<thead>
<tr>
<th>EXPERIMENTAL</th>
<th>119</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction of Hemicelluloses with Aqueous Ethanol</td>
<td>120</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; Ethylene Glycol</td>
<td>120</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; Glycerol</td>
<td>121</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; Aqueous Sodium Hydroxide</td>
<td>122</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot; Other Reagents</td>
<td>123</td>
</tr>
</tbody>
</table>

**Fractionation of the Hemicelluloses with Fehling's Solution** 124

**SUMMARY AND DISCUSSION OF RESULTS** 127
Numerous attempts were made to isolate a hemicellulose containing arabinose residues and distinct from the xylan already investigated. In no case was there found any pronounced increase in the arabinose/non-arabinose ratio on examination of hydrolysates of various fractions. Since many methods were used it is proposed to deal with them very briefly.

The fractions obtained by different fractionation techniques were hydrolysed (p. 52) and then the hydrolysates were examined chromatographically. Chromatogram papers were developed after being sprayed with Spray No. 1 and quantitative determinations of the sugars present in hydrolysates were made by visual inspection of the spots produced on development of the papers. Determinations were facilitated by comparison of the intensities of the spots produced by the arabinose and xylose in the hydrolysates with those produced by the sugars in standard solutions of arabinose and xylose (ratio 1:5, 1:10, 1:20) which had been spotted on the same paper.

The crude oat straw hemicelluloses used in the work were extracted as detailed previously (p. 63).

Sub-headings on the following pages refer to the principal reagents used in the course of the attempted fractionation to be described.
Extraction of Oat Straw Hemicelluloses with Aqueous Ethanol.

A sample (1 g.) of the crude hemicelluloses was heated under reflux for 4 hours with aqueous ethanol (100 ml.; 70%). The product was filtered and the remaining hemicellulosic material was treated in the same way. The extracts were combined and the volume reduced by distillation to 10 ml. On pouring the concentrate into acetone (ca. 100 ml.) a precipitate formed. A hydrolysate of the precipitate was found to contain arabinose/xylose, 1:20.

The above treatment was repeated on a sample (1 g.) of the crude hemicelluloses using firstly 100% ethanol then 90% aqueous ethanol; in neither case was a precipitate produced when the concentrate was added to acetone (as above). The remaining hemicellulosic material was treated with aqueous ethanol (100 ml.; 80%) and the concentrated extract was poured into a mixture of acetone (100 ml.) and a little acetic acid; a flocculent precipitate formed which on hydrolysis gave arabinose and xylose (1:20).

Extraction of Oat Straw Hemicelluloses with Ethylene Glycol.

A sample (2 g.) of the crude hemicelluloses was shaken with neutral ethylene glycol (50 ml.) for a day; there was no apparent dissolution, but, on heating (70°, 4 h), the major part of the material passed into solution while the remaining
material was converted into a gelatinous mass (A). It was found that the addition of acetone (150 ml.) to the solution followed by the addition of chloroform (ca. 20 ml.) resulted in the formation of a thickly gelatinous layer (B). The solution was decanted and a part of the gelatinous material (B) which then remained was dissolved in water (50 ml.) (in which it was readily soluble) and a precipitate (C) was obtained from the resultant solution by the addition of alcohol (ca. 100 ml.). The various fractions were hydrolysed and the hydrolysates examined chromatographically.

<table>
<thead>
<tr>
<th>Found:</th>
<th>Residue A</th>
<th>arabinose/xylose</th>
<th>1:30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitate B</td>
<td>&quot;</td>
<td>1:20</td>
</tr>
<tr>
<td></td>
<td>Precipitate C</td>
<td>&quot;</td>
<td>1:30</td>
</tr>
</tbody>
</table>

**Extraction of Oat Straw Hemicelluloses with Glycerol.**

A sample (2 g.) of the crude hemicelluloses was heated with glycerol (50 ml.; 3 h at 70°). Part of the material passed into solution (A) while a part formed a thickly gelatinous layer (B) and a further part (C) remained apparently unaltered. The mixture was centrifuged and the clear solution (A) decanted. The gel (B) was mechanically separated from the residual matter (C). To a portion (10 ml.) of solution A there was added water (ca. 5 ml.); a precipitate (D) was formed and was centrifuged down. The centrifugate was poured into a mixture of...
acetone (45 ml.) and chloroform (ca. 1 ml.); a precipitate (E) was formed. Hydrolysates of the different fractions were examined.

**Found:**
- Gelatinous layer (B) only xylose detected.
- Residue (C) arabinose/xylose 1:20.
- Precipitate (D) " " 1:15.
- Precipitate (E) " " 1:20.

**Extraction of the Cat Straw Hemicelluloses with Aqueous Sodium Hydroxide.**

Holocellulose (30 g.) and a quantity of glass marbles were placed in a dark-brown bottle and to it there was added sodium hydroxide solution (1.5 l.; 0.1%). Nitrogen was bubbled through the mixture and then, after sealing the bottle, it was rolled mechanically for 18 hours. The residue was filtered off and was treated in the same way with sodium hydroxide solutions (1.5 l.) of progressively increasing concentration (a number of treatments were made at each alkaline concentration). The extracts were acidified with acetic acid, and acetone (ca. 1 l.) was added to each to precipitate the hemicelluloses. The precipitates were hydrolysed and were then examined chromatographically.
None of the above weights is corrected for ash.
The first two precipitates (0.1%, Nos. 1 and 2) were dark-brown and fairly soluble in cold water from which they were only precipitated on the addition of a large volume of acetone (4 vols).

Extraction of the Oat Straw Hemicelluloses with Other Reagents.

Samples of the crude hemicelluloses have been treated with the following reagents at the temperatures, and for the periods, shown: pyridine (70°; 12 h), aqueous pyridine (100°; 6 h: and room temperature; 1 month), water (100°; 2 days), ammonia of different concentrations (room temperature; 1 month), aqueous methanol (40°; 2 days), and aqueous acetone (40°; 2 days). Either there was no detected material extracted from the crude hemicelluloses using these reagents,

<table>
<thead>
<tr>
<th>Concentration of aqueous sodium hydroxide</th>
<th>Number of extractions at each concentration</th>
<th>Arabinose/xylose ratio of ppt. in hydrolysates formed</th>
<th>Weight of ppt. formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1%</td>
<td>1</td>
<td>1:10</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:10</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1:20</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1:20</td>
<td>0.52</td>
</tr>
<tr>
<td>0.5%</td>
<td>1</td>
<td>1:20</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:30</td>
<td>0.42</td>
</tr>
<tr>
<td>1.0%</td>
<td>1</td>
<td>1:20</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:30</td>
<td>0.33</td>
</tr>
<tr>
<td>2.5%</td>
<td>1</td>
<td>1:20</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:20</td>
<td>0.50</td>
</tr>
<tr>
<td>4.0%</td>
<td>1</td>
<td>1:30</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:30</td>
<td>0.20</td>
</tr>
</tbody>
</table>
or the material extracted was not precipitable under the conditions employed, or the precipitate was too slight for it to be worth while examining it.

Fractionation of the Oat Straw Hemicelluloses with Fehling's Solution.

Decomposition of all copper complexes was effected by the addition of hydrochloric acid (2N) to give a normal acid solution.

An alkaline solution of the crude hemicelluloses (5 g.) was treated with an excess of Fehling's solution and the gelatinous precipitate that formed was separated by centrifugation. The precipitate, after dispersion in water, was treated with hydrochloric acid. The moment that the copper complex had apparently completely decomposed the solution was taken to pH 10 by the addition of sodium hydroxide solution (4%) and acetone (2 vols.) was then added. A slight gelatinous precipitate was formed and, after separating it by centrifugation, it was treated with hydrochloric acid for 15 minutes and then acetone (2 vols.) was added. A white precipitate which was produced gave on hydrolysis arabinose and xylose (1:30).

An alkaline solution of the crude hemicelluloses (1 g.) was treated with Fehling's solution as already detailed and the insoluble complex was separated by centrifugation and was rejected. The centrifugate, after being taken to pH 7 by the gradual addition of hydrochloric acid (0.5 N), was divided
into two equal volumes which were treated as follows:

1) Dialyzed against tap-water until free from copper ions. The solution in the dialysis bag was freeze-dried and the solute was then hydrolysed. Xylose was alone detected in the hydrolysate.

2) Dialyzed against tap-water for a day then the volume of the resultant solution was reduced to 50 ml. by freeze-drying. The concentrate was electrodialyzed, with vigorous stirring, until the solution was free from copper ions. The solution on freeze-drying gave a slight white residue which on hydrolysis yielded arabinose and xylose (1:20).

A solution of the crude hemicelluloses (5 g.) in alkali was treated with an excess of Fehling's solution and the gelatinous precipitate that formed was separated by centrifugation. The precipitate, after dispersion in water, was treated with hydrochloric acid. When the complex had apparently just completely decomposed acetone (1/3 vol.) was added and the precipitate which formed was centrifuged down; it gave two layers — an upper, white; and a lower, blue, which were separated mechanically. It should be noted that this 'blue' precipitate was non-gelatinous and of a distinctly different shade from that of the parent complex. The 'blue' precipitate was dispersed as far as possible in water and was then treated for 2 minutes with hydrochloric acid. The solution was taken to pH 4-5 by the addition of aqueous sodium
hydroxide (4%) and precipitation was effected by the addition of acetone. The precipitate was centrifuged down and the 'blue' layer which then separated was dispersed in water, treated for 2 minutes with acid, and then precipitation effected as before. The procedure was carried out four times in all. Following hydrolysis of the final 'blue' precipitate (p.52) the hydrolysate was de-ionised with Amberlite resin IR 120 (H) and was then examined chromatographically (Found: arabinose/xylose, 1:25).
SUMMARY AND DISCUSSION OF RESULTS

In an earlier part (p. 65) details were given of the fractionation of the copper complexes of the crude hemicellulosic material extracted from oat-straw holocellulose. It was then remarked that the percentage of arabinose residues fell during the fractionation from approximately 5.6 to 3.0% and at this latter value stayed constant. During the first fractionation 26.7% of the hemicellulosic material was not recovered and the material which was recovered had then approximately 4.0% of arabinose residues. It was found that when the hemicellulosic material contained 3.0% of arabinose residues then further attempts at its fractionation merely led to a recurrent loss of approximately 12% of the material. If it may be assumed, as appears to be reasonable, that a similar loss of the hemicellulose (containing 3.0% of arabinose residues) took place in the first fractionation then it may be calculated that the other material lost during that fractionation must have comprised ca. 15% of the crude hemicellulosic material and must have contained approximately 16% of arabinose residues. It is apparent therefore that there must have been some molecular species present in the crude hemicellulosic material in which the ratio of the arabinose to the xylose residues was at least 1/5. It is also probably that a number of hemicelluloses were present in the crude xylan and that, in consequence, the arabinose to xylose ratio of the
residues present in one of those hemicelluloses might then be higher than 1 to 5.

Many attempts were made to achieve some degree of separation of the arabinose-rich fraction from the other hemicelluloses, but no method used yielded a fraction sufficiently enriched with respect to its arabinose residue content to be worth while investigating structurally.

The most obvious method of obtaining a separation was one involving the use of Fehling's solution since this already had been shown to lead to some degree of fractionation. Numerous attempts at the isolation of an arabinose-rich fraction from that part of the hemicellulose mixture which did not form an insoluble copper complex failed to yield any positive results. Other attempts were centred around the fractional decomposition of the copper-complex precipitate which was formed on the addition of Fehling's solution to an alkaline solution of the crude hemicellulosic material; again there was no enrichment of the final product with respect to its arabinose content. Attempts were made to fractionally extract hemicelluloses from the crude hemicellulosic material by treating it with Fehling's solution, but again the results were not indicative of the separation of an arabinose-rich fraction. The various methods involving the use of Fehling's solution yielded products ranging in their ratio of arabinose to xylose residues from 1/20 to 1/30.
Examination of different fractions obtained on the treatment of the crude hemicellulosic material with various organic solvents again failed to indicate any evidence of any pronounced enrichment of any of the materials with respect to its arabinose content. Use of aqueous ethanol (p. 35) of different concentrations also failed to lead to the extraction of an arabinose-rich fraction.

The holocellulose of the oat-straw was treated successively with sodium hydroxide solutions of gradually increasing strength. It was found that the lower the strength of the alkali the higher the percentage of arabinose residues in the material extracted. Furthermore successive treatments with alkali of the same strength led to a decrease in the ratio of arabinose to xylose residues in the material recovered from the extracts. Materials were recovered from these extracts in which the above ratio ranged from 1/10 to 1/30. The fractions in which the ratio was 1/10 were highly impure and from difficulty experienced in precipitating them from solution it was concluded that they probably contained hemicelluloses of low molecular weight.
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<td>&quot;Hemicellulase&quot;</td>
<td>147</td>
</tr>
<tr>
<td>The enzymic hydrolysis of oat straw xylan</td>
<td>147</td>
</tr>
<tr>
<td>Preparation of the digests</td>
<td>147</td>
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<td>Investigation of factors affecting xylanase activity</td>
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<tr>
<td>Effect of pre-treatment of &quot;Hemicellulase&quot; with heat.</td>
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<td>Qualitative examination of the enzymic hydrolysate of oat straw xylan</td>
<td>155</td>
</tr>
<tr>
<td>The enzymic hydrolysis of &quot;Hemicellulase&quot;</td>
<td>157</td>
</tr>
<tr>
<td>Action of &quot;Hemicellulase&quot; on Beechwood Hemicellulose A</td>
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<td>Action of &quot;Hemicellulase&quot; on Esparto Grass Araboxylan</td>
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</tr>
</tbody>
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The enzymic activity, and in particular the xylanase activity, of a number of enzyme preparations has been investigated.

Abbreviations and symbols used in the tables.

\[ A = \text{arabinose.} \quad X = \text{xylose.} \quad X_2 = \text{xylobiose.} \]
\[ X_3 = \text{xylotriose.} \quad Gl = \text{glucose.} \quad Ga = \text{galactose.} \]
\[ \text{Ur.} = \text{molecule containing a uronic acid residue.} \quad \text{tr.} = \text{trace.} \]

The symbol \( \neq \) appended to the name of a sugar means that the sugar was identified on paper-chromatograms by comparison of the spot produced by it, after spraying and heating the papers, with that produced by an authentic specimen of the sugar in question.

Methods and Materials.

Buffer solutions.

B.D.H. Universal Buffer Solution was used. In order to facilitate the acidification which is necessary prior to Schaffer-Hartmann determinations the buffer solution was generally used at about one quarter of the recommended dilution. All pH values were checked by glass-electrode pH meter.
Substrates.

The following substrates were used:

1) Xylan 5 from oat straw (p.67).
2) Xylobiose (p.169).
3) Beechwood Hemicellulose A [(100) (p.40)].
4) Esparto grass araboxylan [(97) (p.35)].
5) Inulin from Dahlia tubers (157).
6) Barley glucosan (158).
7) Sucrose.
8) Ivory-nut mannan (159).
9) Malt starch (160).

Substrates were either suspended or dissolved in water; this was perfectly satisfactory for purely qualitative work. Quantitative work on oat-straw xylan was attempted on a suspension of the xylan but the results were inconsistent due to the heterogeneous nature of the substrate. A satisfactory xylan substrate was prepared in the following way: xylan was dissolved in aqueous sodium hydroxide (4%) and the resultant solution was dialysed against tap-water until free from inorganic ions. The colloidal suspension thus prepared was perfectly stable for several months although slight sedimentation took place after five weeks. The mixture was always shaken prior to use. The percentage of xylan in the colloidal solution was determined from time to time by evaporating down a known volume and weighing the residue.
Reducing sugars.

The reducing power of samples of enzymic digests was determined by the method of Schaffer and Hartmann as modified by Stiles (p. 55). Control digests containing no enzyme were always incubated simultaneously with the enzymic digest. It was assumed in calculations that the reducing power was entirely ascribable to xylose; in the quantitative tables given later the reducing power has been converted into the equivalent weight of xylose.

The reducing-power of solutions containing known weights of xylose was determined and a graph plotted relating the volume of thiosulphate consumed in the Schaffer-Hartmann titrations directly to the weight of xylose.

Qualitative paper chromatography.

Paper-chromatographic techniques were employed exclusively. Papers were irrigated with ethyl acetate/pyridine/water (10:3:3-top layer) spots were developed by spraying the papers with saturated aqueous aniline oxalate and then heating the papers in an oven at 110°. The identification of sugars is based solely on chromatographic evidence.

Preparation of Digests.

Details of the preparation of the various digests differed; particulars are given elsewhere. Prior to the addition of the enzyme to buffered solutions of polysaccharides they were heated to the temperature of the incubating oven (35-37°).
**Taka-diastase.**

The taka-diastase was a commercial preparation supplied by Parke, Davis and Co. Ltd. It was contaminated with lactose and was freed from the latter by dialysing an aqueous dispersion of the enzyme preparation against tap-water; the mixture was freeze-dried and the taka-diastase recovered as a felt-like solid.

**Work on oat-straw xylan.**

For quantitative work the digests were prepared by combining the following reagents: colloidal xylan solution (10 ml.), buffer solution (10 ml.), water (25 ml.), and taka-diastase. The digests were contained in rubber-stoppered conical flasks (50 ml.). A layer of toluene (ca. 2 ml.) was added to the digests to minimise the danger of bacterial attack. Further particulars about the preparation of the digests are supplied elsewhere.

Samples (2-5 ml.) were withdrawn from the digests from time to time and the reducing values determined (p.135). The weight of xylan present in 5 ml. samples of the digests was 13.4 mg.; on hydrolysis this would give 15.2 mg. of xylose (assuming that hydrolysis resulted in the 100% conversion of the xylan into xylose). In the following tables the weight of xylose present in 5 ml. samples of the digests is listed together with the calculated percentage degree of hydrolysis of the xylan.
Effect of pH on xylanase activity of taka-diastase.

The digests contained 20 mg. of taka diastase. They were incubated at 35-37°.

TABLE I

<table>
<thead>
<tr>
<th>Period of Incubation (hours)</th>
<th>Reducing sugars (calculated as xylose).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 2</td>
</tr>
<tr>
<td></td>
<td>Wt.</td>
</tr>
<tr>
<td>20</td>
<td>0.9</td>
</tr>
<tr>
<td>68</td>
<td>1.2</td>
</tr>
<tr>
<td>108</td>
<td>2.0</td>
</tr>
<tr>
<td>376</td>
<td>4.6</td>
</tr>
<tr>
<td>964</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Effect of varying the concentration of the taka-diastase.

The digests contained varying amounts of taka diastase (td). The pH of the digests was 5. They were incubated at 35-37°.

TABLE II

<table>
<thead>
<tr>
<th>Period of Incubation (hours)</th>
<th>Reducing sugars (calculated as xylose).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mg. td</td>
</tr>
<tr>
<td></td>
<td>Wt. mg.</td>
</tr>
<tr>
<td>24</td>
<td>0.9</td>
</tr>
<tr>
<td>68</td>
<td>1.1</td>
</tr>
<tr>
<td>19B</td>
<td>3.5</td>
</tr>
<tr>
<td>990</td>
<td>5.3</td>
</tr>
<tr>
<td>1080</td>
<td>5.4</td>
</tr>
</tbody>
</table>
After incubation for eight weeks the digests containing 40 and 70 mg. of taka-diastase were combined, dialysed against tap-water, and the mixture remaining in the dialysis bag then freeze-dried. The white felt-like material thus obtained consisted principally of taka-diastase; samples of it were treated as follows:

1. A sample was heated in a sealed tube with sulphuric acid (2ml.; 1N; 6h; 100°). The reaction-mixture was neutralised with barium carbonate and the barium residues were centrifuged down. Chromatographic examination of the centrifugate showed that in it there were present xylose and arabinose (10:1) and traces of glucose and of galactose.

2. A sample was incubated in a fresh digest with the addition of fresh taka-diastase. After one week the digest was examined chromatographically; a faint trace of xylose was detected.

3. A sample was incubated in a digest containing 7 mg. of "Hemicellulase" (p. 147) at pH 5.2. After a week the digest was examined chromatographically; the products were the same as those found in 1.

**Effect of pretreatment of the taka-diastase with heat or with ultra-violet light**

A basal medium was prepared as for a normal digest but excluding the addition of colloidal xylan solution; it
contained 20 mg. of taka-diastase. The digest was heated at 55° for 30 minutes. The mixture was cooled to 36° and the xylan solution was added. The pH was 5.

A second digest was prepared in a similar manner but instead of heating the mixture it was irradiated with ultra-violet light for 90 minutes.

### TABLE III

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>Reducing sugars (calculated as xylose).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Wt. mg.</td>
</tr>
<tr>
<td>24</td>
<td>0.7</td>
</tr>
<tr>
<td>68</td>
<td>0.9</td>
</tr>
<tr>
<td>191</td>
<td>2.0</td>
</tr>
<tr>
<td>990</td>
<td>5.0</td>
</tr>
<tr>
<td>1080</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Qualitative examination of the xylan/taka-diastase digests.

Chromatographic examination of all of the above digests after eight weeks incubation at 35-37° showed that the end-products were the same in all digests other than that containing taka-diastase which had been irradiated with ultra-violet light. On chromatograms
of the latter, spots were developed [after spraying (Spray No. 1) as in the table.

<table>
<thead>
<tr>
<th>Rg</th>
<th>0.108</th>
<th>0.13</th>
<th>0.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar</td>
<td>?</td>
<td>A ≠</td>
<td>X ≠</td>
</tr>
<tr>
<td>Colour of spot produced.</td>
<td>pink</td>
<td>pink</td>
<td>pink</td>
</tr>
</tbody>
</table>

Approximate percentage: 10 5 85

A quantity of the above ultra-violet treated digest was spotted on a thick-paper (Whatman 3MM) chromatogram and, after irrigating it (Irrigant No. 3), side-strips were cut from the paper and they were sprayed (Spray No. 1) and spots developed in the usual way. An area of paper, believed to contain only one sugar (Rg 0.108), was cut from the central unsprayed strip of the paper and after eluting it with cold water the eluate was evaporated to dryness at 50° under reduced pressure. The residue was heated in a sealed tube with sulphuric acid (ca. 2 ml.; 1N; 100°; 6 h). The reaction-mixture was neutralised with barium carbonate and the solution was then examined chromatographically. Two sugars were detected, one of Rg value 0.15 and the other of Rg 0.13 (Ratio 3 : 1); it is possible that the spot of
Rg 0.13 was produced by arabinose overlapping with the area corresponding to the unknown sugar. A repeat separation and hydrolysis of the material corresponding to the spot of Rg 0.108 gave xylose and arabinose (5 : 1).

The chromatographic examination of all other digests indicated the presence in them of arabinose and of xylose (1 : 20) and of a quantity of material in the region of the starting-line on the chromatograms; this latter material was also observed on chromatogram papers spotted with the digest containing irradiated taka-diastase.

Further qualitative work was carried out on a digest which contained: xylan (10 mg.), water (2 ml.), buffer (1 ml.) and toluene (ca. 1 ml.). The pH was 5.2. The digest was examined chromatographically and sugars with the Rg values in Table V were detected.

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>Rg values of sugars.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No reducing sugars.</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.00-0.009, 0.016?</td>
</tr>
<tr>
<td></td>
<td>0.086 0.094 0.13 0.15</td>
</tr>
<tr>
<td>100-460</td>
<td>0.00-0.008 ? (X4)</td>
</tr>
<tr>
<td></td>
<td>0.029 0.052 0.085</td>
</tr>
<tr>
<td></td>
<td>tr. 1% tr. 7%</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.096 0.13 0.15</td>
</tr>
<tr>
<td>Gl #</td>
<td>A #</td>
</tr>
<tr>
<td>tr.</td>
<td>12% 80%</td>
</tr>
</tbody>
</table>
A chromatogram paper (Whatman 3MM) was heavily spotted with the above digest after it had been incubated for 460 hours. After irrigation the position of reducing sugars on side-strips of the paper was determined in the usual way. The central area of the paper was cut into pieces each of which was believed to contain one of the above sugars (Table V). The papers were eluted with cold water and the solvent was removed from the eluate by freeze-drying. The sugars were hydrolysed in sealed tubes with sulphuric acid (0.5 ml.; 1N; 100°C; 6h). The neutralised hydrolysates were examined chromatographically. The sugars with Rg values 0.000-0.008, 0.014, 0.029, and 0.055 all gave spots corresponding to xylose in their Rg values, while the starting-line material (Rg 0.000-0.008) gave in addition a spot tentatively identified as xylobiose. The hydrolysate of sugar Rg 0.052 was examined but no sugars were detected on the chromatogram paper.

The above procedure for the identification of the sugars present on chromatogram papers was very tedious and time-consuming and a new procedure was therefore devised; it was partially successful. A Whatmann 3MM paper was cut, and lines were drawn on it, as shown on the following page. The above digest which had been incubated for approximately 460 hours was heavily spotted at positions
A and B.

The paper was irrigated (irrigant No. 4) for 48 hours then the paper was cut along cc' and the side-strip sprayed (Spray No. 1) to locate the position of the various sugars. At position D, beneath the furthest travelled sugar, standard sugars were spotted. The line bb' was then lightly sprayed with a dilute solution of taka-diastase in buffer (pH 5.2). The paper was incubated in a moist atmosphere for 4 days at 20°; it was then heated to 120° to destroy the enzymes. It was irrigated at right angles to the former direction of irrigation with Irrigant No. 4. Spraying (Spray No. 1) and heating of the paper after irrigation revealed that the following sugars were present in the enzymic hydrolysates:
### TABLE VI

<table>
<thead>
<tr>
<th>R&lt;sub&gt;g&lt;/sub&gt; value along line cc'</th>
<th>Sugars detected in enzymic hydrolysate.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000-0.008</td>
<td>xylose and other sugars with R&lt;sub&gt;g&lt;/sub&gt; values ranging from 0.000-0.010.</td>
</tr>
<tr>
<td>0.014</td>
<td>xylose and xylobiose.</td>
</tr>
<tr>
<td>0.085</td>
<td>xylose and xylobiose.</td>
</tr>
<tr>
<td>0.029</td>
<td>xylose.</td>
</tr>
</tbody>
</table>

Unfortunately the paper was itself enzymically hydrolysed and the resultant brown streak on the developed paper in the region of glucose made the identification of small amounts of xylobiose impossible and the method was therefore not used again.

**Work on Esparto-grass Arabinoxylan.**

A digest was prepared containing: esparto grass arabinoxylan (10 mg.), water (2 ml.), buffer solution (1 ml.), and toluene (ca. 1 ml.). The pH was 5.2. The digest was examined chromatographically during a period of 460 hours. After 460 hours chromatograms revealed the presence of sugars having the following R<sub>g</sub> values:

0.000-0.008  0.014  0.084  0.088  0.12  0.15  
?  ?  \(X_4\)  \(X_2\)  \(Ca\)  \(A\)  \(X\)

The ratio of arabinose to xylose was 1 : 10.
Enzyme Preparations from

Gladophora Rupestris

An enzyme preparation from Gladophora rupestris and an enriched enzyme preparation from the same source were kindly supplied by Mr. W. Duncan, and Dr. D. J. Manners. Examination of the enzymic hydrolysates of oat-straw xylan and of esparto grass araboxylan showed that they differed markedly in one respect: the former yielded, inter alia, xylose and xylobiose, while the latter yielded only xylobiose. The results are tabulated below.

The digests in all cases contained the following:
enzyme preparation (15 mg.), xylan (30 mg.), water (2 ml.), and buffer (1 ml.; pH 5.2). The digests were covered by a layer of toluene.

Action of the enzyme preparation from Gladophora rupestris on oat straw xylan.

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>Rg values etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-25</td>
<td>nil.</td>
</tr>
<tr>
<td>30</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>Gl ≠</td>
</tr>
<tr>
<td>100</td>
<td>0.00-0.14</td>
</tr>
<tr>
<td></td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>480</td>
<td>As for 100 hours + 0.12</td>
</tr>
<tr>
<td></td>
<td>A ≠</td>
</tr>
</tbody>
</table>
At 480 hours the percentages of the various sugars were estimated to be as follows:

\[
\begin{array}{cccccccc}
R_g & 0.019 & 0.030 & 0.084 & 0.090 & 0.12 & 0.15 \\
\% & \text{ca.} & 2 & 4 & 36 & 2 & 5 & 51
\end{array}
\]

Action of an enzyme preparation from *Cladophora rupestris* on esparto grass araboxylan.

**TABLE VIII**

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>(R_g) values etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.088 (G_1 \neq)</td>
</tr>
<tr>
<td>100</td>
<td>0.000-0.014 0.019 0.028 0.090 (? \ ? \ ? X_3 \ G_1 \neq)</td>
</tr>
<tr>
<td>480</td>
<td>0.000-0.011 0.014 0.028 0.046 0.082 0.088 (? \ X_4 \ ? X_3 \neq\ ? \ ? \ ? \ G_1 \neq)</td>
</tr>
</tbody>
</table>

|                          | tr. 10\% | 10\% | 80\% | tr. |

Action of a more active xylanase preparation from *Cladophora rupestris* on oat straw xylan.

**TABLE IX**

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>(R_g) values etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>408</td>
<td>0.000-0.012 0.020 0.030 0.068 0.085 0.12 0.15 (? \ ? \ X_4 \ X_3 \neq\ ? \ X_2 \neq \ A \neq \ X \neq)</td>
</tr>
</tbody>
</table>

|                          | 2\% | 2\% | 4\% | 38\% | 4\% | 40\% |
Incubation of digests containing emulsin and either oat-straw xylan or esparto grass araboxylan was followed by chromatographic examination of the digests; only faint traces of glucose and of galactose were detected.

"Hemicellulase"

Work has been carried out using a "Hemicellulase" marketed by Lights. The manufacturers are at present unwilling to divulge the source of this enzyme preparation.

The work has involved the determination of the specificity and products of action of the "Hemicellulase" when digested with a number of substrates under different conditions. Attempts have been made to enrich the enzyme preparation with respect to its xylanase activity and to isolate, if possible, xylanases with specific hydrolytic functions. So far no pronounced enrichment of the xylanase activity has been obtained.

The Enzymic Hydrolysis of Oat Straw Xylan.

Preparation of the digests.

A colloidal solution of the xylan (6.86 g./l.) was used. The digests were prepared by combining the following reagents:
buffer solution (10 ml.), water (25 ml.), "Hemicellulase", colloidal xylan solution (5 ml.), and toluene (2 ml.). The toluene was added to inhibit the potential growth of aerobic bacteria.

**Determination of reducing power.**

Samples (2 ml.) of the digests were withdrawn from time to time and their reducing values were determined (p.135). In all the following tables the "Wt." refers to the weight of sugars (calculated as xylose) present in a 2 ml. sample of the digest. It is assumed that the xylan present in the sample would, on hydrolysis, give rise to 100% of xylose.
Investigation of factors affecting xylanase activity.

1. Effect of pH.

A number of digests were prepared in which the pH differed one to another. All digests contained 10·5 mg. of "Hemicellulase".

### TABLE X

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>pH 4</th>
<th>pH 4.5</th>
<th>pH 5</th>
<th>pH 5.5</th>
<th>pH 6</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt. %</td>
<td>wt. %</td>
<td>wt. %</td>
<td>wt. %</td>
<td>wt. %</td>
<td>wt. %</td>
</tr>
<tr>
<td>10</td>
<td>0.15 8.8</td>
<td>0.20 12.4</td>
<td>0.14 8.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>0.41 24.0</td>
<td>0.45 26.4</td>
<td>0.28 16.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>90</td>
<td>-</td>
<td>0.68 39.3</td>
<td>-</td>
<td>0.21 12.4</td>
<td>0.09 5.3</td>
<td>-</td>
</tr>
<tr>
<td>125</td>
<td>0.89 52.0</td>
<td>-</td>
<td>-</td>
<td>0.33 19.4</td>
<td>-</td>
<td>0.17 9.9</td>
</tr>
<tr>
<td>170</td>
<td>-</td>
<td>0.86 50.4</td>
<td>0.62 36.4</td>
<td>-</td>
<td>0.22 12.8</td>
<td>-</td>
</tr>
<tr>
<td>240</td>
<td>-</td>
<td>-</td>
<td>0.83 49.0</td>
<td>-</td>
<td>0.61 35.6</td>
<td>-</td>
</tr>
<tr>
<td>355</td>
<td>1.06 62.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.78 45.6</td>
<td>-</td>
</tr>
<tr>
<td>1440</td>
<td>1.45 85.0</td>
<td>1.45 85.0</td>
<td>1.24 72.5</td>
<td>1.19 69.8</td>
<td>1.18 69.0</td>
<td>0.39 22.8</td>
</tr>
<tr>
<td>2960</td>
<td>1.60 93.6</td>
<td>1.57 92.1</td>
<td>1.40 81.9</td>
<td>-</td>
<td>1.37 80.0</td>
<td>0.57 35.3</td>
</tr>
<tr>
<td>7320</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.55 90.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11880</td>
<td>1.67 98.0</td>
<td>1.66 97.2</td>
<td>1.59 92.5</td>
<td>1.59 93.0</td>
<td>1.56 91.5</td>
<td>0.70 40.8</td>
</tr>
<tr>
<td>18720</td>
<td>1.72 100.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.61 94.0</td>
<td>0.86 50.2</td>
</tr>
</tbody>
</table>
2. Effect of concentration of xylanase.

Two digests were prepared one (A) containing 18.4 mg. of "Hemicellulase"; the other (B) 1.4 mg. The pH was adjusted to 4.5.

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>Reducing sugars (calculated as xylose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Wt. mg. %</td>
</tr>
<tr>
<td>10</td>
<td>0.09 5.3</td>
</tr>
<tr>
<td>34</td>
<td>0.22 12.9</td>
</tr>
<tr>
<td>120</td>
<td>0.40 23.4</td>
</tr>
<tr>
<td>334</td>
<td>-</td>
</tr>
<tr>
<td>1380</td>
<td>0.77 45.0</td>
</tr>
<tr>
<td>4560</td>
<td>-</td>
</tr>
<tr>
<td>10,080</td>
<td>-</td>
</tr>
</tbody>
</table>

3. Effect of pretreatment of "Hemicellulase" with heat.

Three basal media were prepared as for a digest but without the addition of the xylan solution; "Hemicellulase" (20.4 mg.) was present in each. The digests were heated for the following periods and at the following temperatures: 45° for 1 hour (A'); 61° for 20 hours (B'); and 75° for 20 hours (C'). The digests were then cooled to 36° and a colloidal solution of xylan (pre-heated to 36°) was then added. The flasks were incubated at 35-37°. The pH of the digests was 4.5.
Chromatographic examination of the digests after 3 days' incubation showed that the first two (A' and B') contained arabinose and xylose in the ratio of 1 to 20. Arabinose was detected in digest C', but there was no evidence of any xylose being present.

4. Effect of pretreatment of "Hemicellulase" with ultra-violet light.

A basal medium was prepared as for a digest but excluding the addition of the colloidal xylan solution; it contained 22.4 mg. of "Hemicellulase". The mixture was irradiated with ultra-violet light from a Hanovia lamp for two hours. During the irradiation the temperature of the mixture rose to 45°. The mixture was allowed to cool to 36° and xylan solution (preheated to 36°) was then added.

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>Reducing sugars (calculated as xylose).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A'</td>
</tr>
<tr>
<td></td>
<td>Wt. mg.</td>
</tr>
<tr>
<td>10</td>
<td>0.22</td>
</tr>
<tr>
<td>34</td>
<td>0.42</td>
</tr>
<tr>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>334</td>
<td>-</td>
</tr>
<tr>
<td>1,330</td>
<td>1.27</td>
</tr>
<tr>
<td>4,560</td>
<td>1.55</td>
</tr>
<tr>
<td>10,080</td>
<td>1.50</td>
</tr>
</tbody>
</table>
pH of the digest was 4.5. Details are given in column D' of Table XII.

Chromatographic examination of this digest after three days' incubation indicated that the arabinose/xylose ratio was 1 to 20 - the same as that in a similar digest containing non-irradiated "Hemicellulase".

5. Effect of dialysis of "Hemicellulase" solution on its xylanase activity.

A sample (50 mg.) of "Hemicellulase" was shaken with water (100 ml.) and the resultant mixture was dialysed against running tap-water for a few days then against successive volumes of distilled water for a week. The dialysed mixture was then freeze-dried and a sample (10.5 mg.) of the recovered "Hemicellulase" was incorporated in a digest prepared in the normal way (pH 4.5).

```
<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>10</th>
<th>27</th>
<th>162</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing sugars (calculated as xylose).</td>
<td>Wt. mg.</td>
<td>0.17</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>10.0</td>
<td>16.9</td>
</tr>
</tbody>
</table>
```

Chromatographic examination of the digest after incubation for a week, indicated that the hydrolysis products were the same, and in the same proportions, as those in a digest containing non-dialysed "Hemicellulase".
6. Effect of the addition of small amounts of salts of heavy metals to the digests.

Four digests were prepared in the usual way each containing 10.5 mg. of "Hemicellulase". The pH was 4.5. A small quantity (ca. 5 mg.) of one of the following salts was added to each digest: copper sulphate (A), cadmium sulphate (B), lead acetate (C), and barium chloride (D).

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>Reducing sugars (calculated as xylose).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Wt. mg.</td>
</tr>
<tr>
<td>25</td>
<td>0.34</td>
</tr>
<tr>
<td>167</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Chromatographic examination of the digests after incubation for a week indicated that they were not significantly different from a digest containing no heavy metal salts; the chromatograms were not very satisfactory as, due to the presence of the ions, there was some 'streaking' of the spots.

7. Comparison of the xylanase activities of the 'soluble' and 'insoluble' fractions of the "Hemicellulase".

It was noted that when a sample (50 mg.) of the "Hemicellulase" was shaken with water (20 ml.) part of the material readily dispersed, while a part remained as a
residue. Digests were prepared one containing 10·5 mg. of 'soluble' "Hemicellulase" and the other the same weight of the 'insoluble' fraction (pH 4·5).

TABLE XV

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>Reducing sugars (calculated as xylose).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Wt. mg.</td>
</tr>
<tr>
<td>8</td>
<td>0·20</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>0·56</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromatographic examination of the two digest indicated that the two "Hemicellulase" fractions were not significantly different in their xylanase activities.

8. Effect of alcoholic precipitation of the 'soluble' "Hemicellulase".

A sample (50 mg.) of the "Hemicellulase" was shaken with water (20 ml.) and the 'insoluble' residue centrifuged down. To the centrifugate there was added the minimum volume of alcohol (ca. 20 ml.) necessary to cause a slight precipitation. The precipitate was centrifuged down and separated. It was dried in vacuo at 35°. A digest was prepared containing 10·5 mg. of the precipitated "Hemicellulase". The pH was adjusted to 4·5.
Chromatographic examination of the digest after 3 days' incubation indicated that the hydrolysis products were present in the same proportions as in a digest incubated with non-precipitated "Hemicellulase".

**Qualitative examination of the enzymic hydrolysate of oat-straw xylan.**

Three qualitative investigations of the action of "Hemicellulase" on oat-straw xylan have been carried out.

a) The digest contained colloidal solution of xylan (6.86 g./l.; 3 ml.), "Hemicellulase" (10 mg.), and buffer solution (1 ml.). The pH of the digest was 4.2. Chromatogram papers were very heavily spotted with samples of the digest; it took about four minutes to apply each series of spots.
**TABLE XVII**

<table>
<thead>
<tr>
<th>Period of Incubation (minutes)</th>
<th>Rg values and ratios of sugars in the digest.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>? Ur</td>
</tr>
<tr>
<td>3-7</td>
<td>less than 1</td>
</tr>
<tr>
<td>7-11</td>
<td>&quot;</td>
</tr>
<tr>
<td>11-15</td>
<td>&quot;</td>
</tr>
<tr>
<td>15-19</td>
<td>&quot;</td>
</tr>
<tr>
<td>19-23</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The amount of arabinose present remained constant throughout the 23 minutes.

b) The digest was prepared as in (a). The pH was 5.3. Chromatogram papers were lightly spotted with samples of the digest.

**TABLE XVIII**

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>Rg values and percentages of sugars in the digest.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>? Ur</td>
</tr>
<tr>
<td>420</td>
<td>1%</td>
</tr>
<tr>
<td>3,900</td>
<td>1%</td>
</tr>
</tbody>
</table>

c) The digest was prepared as in (a). The pH was 5.3. Chromatogram papers were heavily spotted with samples of the digest.
<table>
<thead>
<tr>
<th>Period of Incubation (minutes)</th>
<th>( R_g ) values and percentages of sugars in the digest.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.010</td>
</tr>
<tr>
<td>90</td>
<td>3%</td>
</tr>
<tr>
<td>210</td>
<td>11%</td>
</tr>
<tr>
<td>360</td>
<td>8%</td>
</tr>
<tr>
<td>1,440</td>
<td>1%</td>
</tr>
</tbody>
</table>

The enzymic hydrolysis of "Hemicellulase".

A digest was prepared containing the following:
colloidal solution of xylan (6.86 g./l.; 3 ml.), papain (10 mg.), "Hemicellulase" (10 mg.), buffer solution (1 ml.) and water (2 ml.). The pH was 4.2. After incubation for 5 days a sample of the digest was examined chromatographically; spots with the following \( R_g \) values were observed on the paper:

\[
\begin{array}{cccccc}
R_g & 0.016 & 0.030 & 0.078 & 0.15 \\
\text{tr.} & ?X_4 & ?X_3 & X_2 & X \\
\end{array}
\]

In separate experiments it was determined (i) that the papain had no xylanase activity and (ii) that the preparation used did not itself give rise to any spots on chromatogram papers when they were sprayed with aniline oxalate and then heated.
Another digest was prepared containing the following: papain (100 mg.), "Hemicellulase" (100 mg.), water (10 ml.), and buffer solution (3 ml.). The pH was again 4.2. After digestion for one week the flask was well shaken and a sample (approximately 1/10th of the contents) was removed and added to another flask containing: buffer solution (1 ml.), colloidal xylan solution (2 ml.). The digest had a pH of 4.2. It was incubated for one week and then a sample withdrawn and examined chromatographically. Sugars with the following $R_g$ values were detected:

$$R_g \begin{array}{cccc} 0.081 & 0.15 \\ X_2 & X \\ 70\% & 30\% \end{array}$$

**Action of "Hemicellulase" on Beechwood Hemicellulose A.**

A digest was prepared containing: buffer solution (10 ml.), water (30 ml.), "Hemicellulase" (10 mg.), beechwood xylan (31.5 mg.) and toluene (2 ml.). The pH was 4.5. From time to time 2 ml. samples were withdrawn and their reducing values determined.

### TABLE XX

<table>
<thead>
<tr>
<th>Period of incubation (minutes)</th>
<th>10</th>
<th>40</th>
<th>110</th>
<th>452</th>
<th>2,256</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing sugars (calculated as xylose).</td>
<td>mg.</td>
<td>0.18</td>
<td>0.40</td>
<td>0.31</td>
<td>1.10</td>
</tr>
<tr>
<td>%</td>
<td>10.1</td>
<td>22.4</td>
<td>17.4</td>
<td>61.7</td>
<td>92.6</td>
</tr>
</tbody>
</table>
Samples of the digest were examined on paper chromatograms; only xylose and xylobiose were detected in the early stages of the hydrolysis while later only xylose was found.

**Action of "Hemicellulase" on Esparto Grass Arabinoxylan.**

A digest was prepared, as in the previous section, containing Esparto Grass arabinoxylan instead of Beechwood hemicellulose A. Chromatographic examination of a sample of the digest after 40 minutes incubation showed that in it arabinose and xylose were present in the ratio of 1:4, while after 24 hours this had fallen to 1:6.

**The Action of "Hemicellulase" on Substrates other than Xylan.**

**Preparation of the digest.**

Digests contained the following reagents: buffer solution (1 ml.), "Hemicellulase" (4 mg.), and either (a) a solution of the saccharide (2 ml., 1%), or (b) the saccharide (20 mg.) shaken into suspension in water (2 ml.). The digests were adjusted to pH 4.2.

**Results.**

In the summary of results following the period of incubation is quoted and is followed by the $R_x$ values of the different sugars determined after irrigation of paper-chromatograms spotted with samples of the digests. The papers were irrigated for 48 hours with Irrigant No. 4.
\[ R_x = R_{xylose} \]

Malt starch.

7, 24, and 65 hours: \( R_x 0.58 \)

Inulin from Dahlia tubers.

40 mins. \( R_x 0.060 \) 0.75 fructose ≠

80% 20%

24 hours. \( R_x 0.79 \) fructose ≠

Ivory-nut mannan.

7 hours. \( R_x 0.082 \) 0.22 0.76 mannose ≠

5% 15% 80%

24 hours. \( R_x 0.22 \) 0.48 0.56 0.73 Gl ≠ mannose ≠

After 24 hours the ratio of sugar \( R_x 0.22 \) (mannobiose ?) to mannose was 1:20.

Barley glucosan.

24 hours. \( R_x 0.60 \) gl. ≠

Sucrose.

7 hours. \( R_x 0.060 \) 0.080 Gl ? fructose ?

Digests were prepared with the following modifications:

1) The "Hemicellulase" was heated at 60° for two hours in the digest mixture prior to the addition of the substrate.
2) A number of digests were prepared each containing a little (1-2 mg.) of one of the following salts: copper sulphate, cadmium sulphate, barium nitrate and lead acetate. The digests were not de-ionised prior to their chromatographic examination and as a result some of the papers were difficult to interpret due to 'streaking' of the spots.

3) The "Hemicellulase" was dialysed against tap-water then against distilled water (p. 152) prior to its addition to the digest.

In no case was there any apparent difference in the enzymic activity of the "Hemicellulase" with respect to the above substrates.

Digests were prepared containing high concentrations of the following single sugars as substrates: xylose, glucose, and arabinose. Other digests were prepared with these pairs of sugars as substrates: arabinose and xylose; arabinose and xylobiose; and xylobiose and glucose. Chromatographic examination of these digests after periods ranging from 30 minutes to a week gave no indication of the presence of any unexpected sugars. The only reaction appeared to be the hydrolysis of the xylobiose to xylose.

Fractionation of "Hemicellulase".

Since the "Hemicellulase" had a high xylanase activity attempts were concentrated on the small scale fractionation
of the mixture of enzymes by chromatographic and ionophoretic techniques.

The detection of enzymes on paper.

A primary consideration in the use of paper chromatograms and ionophoretograms for the separation of the enzymes was that of finding some reagent by means of which the position of the various enzyme fractions could be located. Much work centred on this objective but led to few satisfactorily positive results.

Many recommended methods were tried but few were of any use. Durrum, and Kunkel and Tiselius (161) used an alcoholic solution of bromophenol blue for the detection of proteins on paper ionophoretograms. The method was the most successful of those investigated by the author, for the non-specific detection of proteins, but it was far from satisfactory as it required relatively high concentrations of protein. Papers (Whatman No. 1) carrying enzymes were dried and were then soaked for a period of 15 minutes in the bromophenol blue reagent. The papers were removed and washed thoroughly with running cold water. When the papers were steeped in the reagent an area of the paper was often stained grey-blue; washing with water normally led to the complete decolouration of the stained area and sometimes to the production of a stain in another area: the two areas were always
noted. For some unknown reason the sensitivity of this staining technique was very greatly reduced when Whatman No. 3MM paper was used instead of Whatman No. 1 paper: this in spite of the fact that the former papers were, proportionate to the latter, always much more heavily spotted with the enzymic mixture.

Two methods were devised for the detection of enzymes, as opposed to proteins, on paper chromatograms and ionophoretograms. In the first method the dry enzyme-carrying paper was lightly sprayed with a colloidal solution of xylan \((\text{ca. } 10 \text{ g./l.})\). It was then incubated for several days at 37° in an atmosphere saturated with water vapour. The paper was removed, dried, and then sprayed with saturated aqueous aniline oxalate; it was heated in an oven at 110° to reveal the position of any reducing sugars. This method was applied for the detection of the position of xylanases. It had the limitation that cellulases present in the "Hemicellulase" hydrolysed the paper and these hydrolysis products on treatment with aniline oxalate produced a background colouration.

Another, and a more satisfactory, method used was to spray a strip of Whatman No. 1 paper repeatedly with the colloidal xylan solution with intermediate drying. The xylan-paper was then sprayed lightly with buffer solution and the paper placed on top of a dry enzyme-carrying paper. The two papers were clamped together
between glass plates and incubated at 20° in an atmosphere saturated with water vapour. After several days the two papers were removed, dried and treated with aniline oxalate solution as above. The method had the disadvantage that it required rather large quantities of xylan for the impregnation of the paper.

When these methods failed recourse was made to the digestion of xylan solution either with material eluted from different areas of the paper with water, or to its digestion together with the areas of paper on which enzymes were believed to be present. The digestes were, after a period of incubation at 37°, examined chromatographically for reducing sugars.

Attempts to separate the constituents of "Hemicellulase" by ionophoretic techniques.

The apparatus used and the techniques employed have been described (p. 53). On to a line drawn centrally across the width of a strip (3-10 cm.) of Whatman No. 1 paper there was spotted a small amount of a centrifuged aqueous colloidal solution of "Hemicellulase". The spot was dried with cold air and the spotting procedure repeated several times. The ends of the paper-strip were placed in a bath of buffer solution and the solution was allowed to soak up to within 1 cm. on either side of the central line. The paper was evenly and well blotted and was then placed on a glass plate with the
ends of the paper hanging into two electrode-compartment containing the same buffer solution as on the paper. A piece of buffer-moistened paper was placed on top of the dry part of the paper-strip and when the latter was moistened the former was removed. A glass plate was clamped on top of the paper-strip and a potential difference applied between the ends of the paper.

In different experiments borate, phosphate, and citrate buffers were used; the pH was varied from 4 to 8, the voltage from 200 to 1,200, and the time of the run from 30 minutes to 2 days. The distances moved by the proteins varied very much even when apparently the same conditions were being employed; probably the variable factor was the degree of moistness of the paper. The best separations achieved were when the following conditions were used: pH 6.9 (phosphate buffer) and 200 volts for 1 hour. The position of the enzymes was determined by the xylan-paper technique (p.163). The xylan-paper was sprayed with buffer of pH 3 in order to reduce the pH of the ionophoretogram when the two were placed in contact. After incubation and aniline oxalate treatments two spots were observed on one paper, one displaced about 4 cm. from the central line in the direction of the cathodic end of the paper, the other displaced 2 cm. in the opposite direction. The first spot was brown, the second pink indicative of their being produced by hexose and pentose
respectively. A second separation was carried out on a fresh ionophoretogram in exactly the same manner. The paper was removed from the ionophoresis apparatus dried, sprayed with buffer solution (pH 3), and then incubated at 37° for a few days. Aniline oxalate treatment of the dried paper indicated the presence of a cellulase toward the cathodic end of the paper and about 3 cm. from the starting line; this was presumably the same spot as noted on the earlier ionophoretogram.

Attempts were made to increase the separation of the enzyme fractions by increasing the time of the ionophoresis run (up to 2 days), by increasing the potential difference between the ends of the paper (up to 1,200 volts), and by varying the pH of the impregnating buffer solution. Increasing either the time of the run or the potential difference resulted in an inability thereafter to develop any spots by any of the above techniques.

Attempts to separate the constituents of "Hemicellulase" by chromatographic techniques.

Attempts have been made to fractionate the enzymes on paper chromatograms irrigated with various mixtures of acetone and water. Following irrigation the papers were dried and then treated with bromophenol blue reagent (p. 162). It was found that, depending on the ratio of the acetone to the water, the protein either did not move at all or entirely moved with the solvent front.
Aqueous solutions of ammonium sulphate of different degrees of saturation were used for the irrigation of a series of papers by both upward and downward irrigation techniques. The pH of the solutions was adjusted by the addition of sulphuric acid or sodium hydroxide. Papers were normally irrigated at a temperature of approximately 15-20°.

Thirty-six papers were irrigated under varying conditions. It was found that degrees of saturation from 1/4 to 1/2 resulted in the production, on treatment of the papers with bromophenol blue reagent, of an elongated streak which appeared to consist of a number of interlinked spots. The maximum length of the streak was obtained when the pH was 8.7 and the solution was 1/3rd saturated. On papers irrigated with solutions of a lower saturation than 1/4 the protein travelled with the solvent front; on papers irrigated with solution more than 1/2 saturated the protein did not move from its initial position.

A large number of papers (5 x 45 cm.; Whatman No. 1) were irrigated with 1/3rd saturated aqueous ammonium sulphate solution of pH 8.7. One of the papers, after upward irrigation for ten hours, was treated with bromophenol blue reagent: a streak 20 cm. long was produced. The unstained chromatograms (6 of 2 x 40 cm.) were cut so as to obtain pieces of paper corresponding to 4 cm. lengths
of the streak; matching papers from different chromatograms were combined. The papers were shaken with water and the eluates freeze dried.

Digests were prepared (p.135) containing buffered xylan solution and the enzymes extracted from the papers. Samples of the digests were examined after they had been incubated for various periods; xylose alone was noted. Larger quantities of the enzymes must be separated and this is being attempted by using a chromatopile (l62) irrigated with the above ammonium sulphate solution.
APPENDIX

Preparation and isolation of xylobiose and of xylotriose.

A quantity (60 g.) of the arabinoxylan isolated from esparto-grass by Aspinall, Hirst, Moody and Percival (p. 35) was shaken for four days with water (500 ml.) and the volume was then made up to 2 litres. A sample (100 ml.) was withdrawn and sulphuric acid (25 ml.; 2N) was added to it and the mixture was heated on a boiling water-bath during a period of 190 minutes. From time to time samples were withdrawn and after neutralisation were examined chromatographically (Irrigant No. 6; Spray No. 1) and spots were developed by heating the sprayed papers. The ratios of the various sugars present in the hydrolysate were estimated approximately, by visual inspection of the spots, to be as follows:

<table>
<thead>
<tr>
<th>Time from start of hydrolysis (minutes)</th>
<th>X₃</th>
<th>X₂</th>
<th>X</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.2</td>
<td>0.2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>70</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>90</td>
<td>0.5</td>
<td>1.1</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>110</td>
<td>0.5</td>
<td>1.0</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>130</td>
<td>0.5</td>
<td>1.0</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>160</td>
<td>0.3</td>
<td>0.3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>190</td>
<td>tr.</td>
<td>0.5</td>
<td>6.5</td>
<td>1</td>
</tr>
</tbody>
</table>

169
It was decided that a good yield of $X_2$, of $X_3$ and possibly of higher oligosaccharides would best be obtained after a period of 80 minutes hydrolysis under the same conditions. The remaining aqueous mixture of xylan (1,900 ml.) was acidified and was then heated as before while being vigorously stirred. After 80 minutes the mixture was quickly cooled to 5° and sodium bicarbonate was added until the mixture was alkaline.

A column (68 mm x 780 mm) of charcoal/celite (50:50 by wt.) was prepared by the method recommended by Whistler and Durso and the filtrate from the above alkaline mixture was passed through the column which was then washed with water for a fortnight by which time the eluate was free from xylose. The column was successively irrigated with aqueous ethanol of the following concentrations and during the following times: 3% (5 weeks), 7% (5 weeks), and 10% (15 weeks). The 3 and 7% aqueous alcohol eluates were found to contain xylobiose which was slightly contaminated with xylose while the 10% eluate contained xylotriose which was slightly contaminated with xylobiose.
SUMMARY AND DISCUSSION OF RESULTS

During the work five different enzyme preparations were studied for evidence of xylanase activity; the preparations were:

1) a commercial taka-diastase.
2) a commercial emulsin.
3) two enzyme preparations from Cladophora rupestris.
4) a commercial preparation called "Hemicellulase".

The taka-diastase had xylanase activity but was slow in its catalytic action. The enzyme preparations from Cladophora rupestris had xylanase activity but were in short supply and work on them, although promising, had to be abandoned. The emulsin had no xylanase activity.

The "Hemicellulase" has been most fully investigated; it had xylanase activity, but, as will be seen later, it was a mixture of enzymes; attempts were made to separate them.

Xylans were incubated in buffered solutions with the above enzyme preparations. The products of enzymic action and the reducing power of samples of the digests were determined from time to time. Reducing power was determined by the method of Schaffer and Hartmann and the weight of xylose of equivalent reducing power was then calculated. In the calculation the assumption is made that the reduction of the Schaffer-Hartmann reagent was due to xylose alone,
but since arabinose, xylo-oligosaccharides, and molecules containing uronic acid residues were at times all present in the enzymic hydrolysates this assumption is only a convenient approximation. As hydrolysis proceeded the amount of xylose in the various digests increased and the above assumption then became more readily justifiable. In any case the above measurement of the reducing power was a measure of the overall rate of hydrolysis. From the calculated weights of xylose present in samples of digests the percentage degree of hydrolysis of the xylan was determined; the above limiting considerations again must be borne in mind.

**Taka-diastase.**

The xylanase activity of the taka-diastase was most pronounced at pH 4.5. It was determined that, depending on the concentration of the taka-diastase in the digests, and under the conditions studied, the final degree of hydrolysis of oat straw xylan ranged from 35.5 to 46.0%. A polysaccharide (A) which was isolated from these digests was not attacked on its inclusion in a fresh digest together with taka-diastase. The polysaccharide A was however hydrolysed on its inclusion in a digest together with "Hemicellulase" and the products then were xylose and arabinose (10:1); the same products were obtained on the acid-hydrolysis of another sample of polysaccharide A.
The isolation of such a polysaccharide gives support to the structure proposed for the xylan in which arabinose was stated to be an integral part of the molecule. The reason for the cessation of hydrolysis of the xylan is problematical. The simple explanation that it is due to a side-chain blocking the stepwise hydrolysis of the glycosidic bonds in the xylan appears to be untenable when it is noted that, in the earlier stages of hydrolysis, there are produced xylo-oligosaccharides indicative of the enzyme reaction involving the random and not the stepwise hydrolysis of glycosidic bonds.

Heating of the taka-diastase led to a slight decrease in its enzymic activity, but there was no qualitative difference in the products of the hydrolysis compared with those found after incubation of the xylan with unheated enzyme. This was not the case when the enzyme preparation was irradiated with ultra-violet light; there was then a considerable loss of xylanase activity and a new, or at any rate a hitherto undetected, sugar was noted on chromatographic study of the hydrolysate. This sugar may be an oligosaccharide containing xylose and arabinose residues in the ratio of 5:1; the evidence is however not satisfactory.

Incubation of a buffered solution of esparto grass araboxylan with the taka-diastase was followed by the chromatographic examination of the products; they were
qualitatively similar to those found in enzymic hydrolysates of oat-straw xylan.

**Enzyme Preparations from Cladophora Rupestris.**

Both enzyme preparations from *Cladophora rupestris* possessed xylanase activity; one was rather more active than the other. The less active preparation catalysed the hydrolysis of oat straw xylan giving as principal end-products xylose and arabinose, but the enzyme preparation on incubation with esparto grass araboxylan gave rise neither to xylose nor to arabinose; the principal product was in this case xylobiose. Without further study it would be futile to formulate any theory regarding the nature of the enzyme action, or regarding the fundamental difference in the structure of the two substrates, in order to account for the difference in the products of enzymic hydrolysis. Due to the shortage of the enzyme preparations work had unfortunately to be discontinued.

"Hemicellulase".

The ascription of the term "Hemicellulase" to the enzyme preparation supplied by Lights was a somewhat unfortunate misnomer since it implied that the preparation was specific for the hydrolysis of hemicelluloses only. The preparation, in addition to catalysing the hydrolysis of xylan, araboxylan, and mannann, also catalysed the hydrolysis...
of the non-hemicelluloses; glucosan, starch, inulin, cellulose, and sucrose. The principal glycosidic linkages in those molecules were: α and β 1→4 glucopyranosidic, β 1→4 manno-pyranosidic, β 1→2 fructofuranosidic, β 1→4 xylopyranosidic, and also the 1→3 link between arabofuranosidic and xylopyranosidic residues, and the 1→1 link between glucopyranosidic and fructofuranosidic residues.

There was a possibility that in the "Hemicellulase" there might be transglycosylases present, that is, enzymes present which could catalyse the transfer of a glycosyl residue from one molecule to another. There was, however, no evidence of transxylosylation having taken place during the incubation of digests containing, as substrates, either xylobiose and arabinose, or xylobiose and glucose. This and other work also indicated that there was no evidence that any of the enzymes present possessed synthetic activity and could, for example, synthesise disaccharides from monosaccharides.

The action of "Hemicellulase" on oat-straw xylan was studied both qualitatively and quantitatively and from this study it was concluded that there were probably two xylanases present in the "Hemicellulase". The considerations that led to this conclusion will be stated shortly. One of the enzymes present was believed to catalyse the random hydrolysis of the xylan and to give xylobiose as the principal end-product;
this enzyme is hereafter called Xylanase A. The other xylanase believed to be present catalysed the hydrolysis of xylobiose to xylose and may have had other xylanase activity; this xylobiase is hereafter called Xylanase B.

There are at least three ways in which the enzymic hydrolysis of xylan could proceed:

1) By the scission of single residues from either the reducing or non-reducing ends of the chains of xylose residues or possibly from both ends. Such hydrolysis would give xylose as the principal product.

2) By the random hydrolysis of the molecule to give molecules of varying molecular weights. In the early stages of such a hydrolysis a mixture of xylo-oligosaccharides would be found in the hydrolysate.

3) By the stepwise scission of molecules of a definite size possessing more than one xylose residue; the attack would again probably take place from the ends of the molecular chains.

Chromatographic examination of "Hemicellulase"-xylan digests indicated the presence in them, during the early stages of hydrolysis, of xylose, xylobiose, xylotriose and possibly of xylotetrose and higher oligosaccharides. The hydrolysis would therefore appear to have taken place by the second mechanism; although the possibility that more
than one enzyme may have been involved in the initial stages of the hydrolysis should not be discounted.

It was found (Fig. 4.) that the optimum pH for the hydrolysis of xylan was 4. During the investigation of the effect of the hydrogen ion concentration on the xylanase activity of the "Hemicellulase" it was found that at pH 4-6 the hydrolysis went ultimately to completion or near to completion. Chromatographic examination of digests in which the xylan was believed to be hydrolysed to the extent of about 50% indicated that xylose and xylobiose were present in the digests in the approximate ratio of 2:1. On the accompanying figure (Fig. 4) the degree of hydrolysis of xylan is plotted against the period of incubation of the digest. It will be seen that in digests which were buffered to pH 4-6 there were apparently abrupt changes in the rates of reaction after 40-50% of the xylan had been hydrolysed. In those enzymic hydrolysates the principal sugars were xylobiose and xylose. If the postulation that the Xylanase A does not hydrolyse xylobiose is correct then it is suggested that the break in the curves may correspond to the cessation or near cessation of the activity of Xylanase A. The continued increase in the reducing power of the digests beyond this point is attributed to the action of Xylanase B which was, presumably, also active at earlier stages of the hydrolyses. In summary: the first part of the curve is postulated to be a reflection of the action of two xylanases, and the latter part of only one (Xylanase B).
DEGREE OF HYDROLYSIS OF XYLAN
AGAINST THE PERIOD OF
INCUBATION OF THE DIGEST.

Fig. 4.
Further evidence in favour of the theory of the existence of Xylanase A and B was supplied when a sample of "Hemicellulase" was incubated in a buffered solution with papain and xylan. Papain is a proteolytic enzyme and it was hoped that it would preferentially catalyse the hydrolysis of one or other of the above Xylanases. Chromatographic examination of the digest after a period which, in the absence of loss of xylanase activity by the "Hemicellulase", would normally have led to the complete hydrolysis of the xylan, showed that the hydrolysate contained xylobiose (80%), xylose (10%), and xylotriose (10%). This indicated that the papain had preferentially destroyed a xylobiase (Xylanase B) whilst leaving the Xylanase A activity largely or entirely unimpaired. Subsequent work with the enzymic product of papain-"Hemicellulase" digests provided further evidence in favour of this view.

The "Hemicellulases" appeared to attack beechwood xylan and esparto grass araboxylan in a similar way to oat straw xylan. Under the conditions normally employed the arabinose side-chains present in the oat straw xylan were completely removed by hydrolysis within a few minutes of the start of the reaction. It was found that when the Xylanase A and B activities of the "Hemicellulase" had been apparently completely destroyed by heating the enzyme preparation, the product still retained its ability to catalyse the hydrolysis of the arabinose side-chains.
Other methods were employed in an endeavour to modify the enzymic activities of the "Hemicellulase". It was found that the addition of salts of heavy metals did not apparently lead to any such modification. A solution of the "Hemicellulase" was dialysed against distilled water in an endeavour to remove possibly-existing co-enzymes and it was found that the recovered and the original preparations were apparently enzymically identical. Neither irradiation of the "Hemicellulase" with ultraviolet light nor its precipitation from aqueous solution by the addition of alcohol led to any significant alteration in its enzymic properties.

Attempts were made to separate enzymes with specific xylan hydrolytic functions from the "Hemicellulase". The attempts were at first concentrated on the fractionation of small amounts of the "Hemicellulase" on paper by chromatographic and ionophoretic techniques. Difficulty was experienced in locating the position of enzymes on paper chromatograms and ionophoretograms. A bromophenol blue reagent was used in order to stain proteins present on the papers but it was insufficiently sensitive and was in any case entirely non-specific for the detection of individual enzymes. More satisfactory results were obtained by incubating papers on which enzymes were present with buffered solutions of xylan and locating
any reducing sugars produced by spraying the papers with a solution of aniline oxalate and then developing spots by heating.

By an ionophoretic technique, combined with the above enzyme-detection technique, it was determined that an enzyme possessing cellulase but no xylanase activity and an enzyme possessing xylanase but no cellulase activity were present in the "Hemicellulase". The separation of those two enzymes on paper ionophoretograms was slight, but attempts to increase it by using more drastic conditions resulted in an inability thereafter to detect any enzymes and it is therefore assumed that they were denatured.

Unsuccessful attempts were made to fractionate the "Hemicellulase" on paper chromatograms irrigated with various concentrations of aqueous acetone. More recently aqueous solutions of ammonium sulphate have been used as irrigants and the results so far obtained suggest that the method leads to some fractionation of the "Hemicellulase".
A Xylan from Oat Straw.

1. Hemicelluloses were extracted with dilute alkali from oat straw holocellulose. The hemicellulosic material (yield 19% on weight of dry straw) had:
arabinose residues/xylose residues, 1:17; \([\alpha]_D^{18} -94.7^\circ (c, 0.6 \text{ in } \text{N NaOH}).\)

2. Fractionation of the hemicelluloses, by treatment with Fehling's solution, yielded a xylan which had: arabinose residue content, 3.0%; OMe content, 0.5%; lignin content, 3.3%; ash, 0.8%; uronic anhydride, 3.5%; and \([\alpha]_D^9 -95.0^\circ (c, 0.5 \text{ in } \text{N NaOH}).\)

3. The xylan was methylated and the product was fractionally dissolved in mixtures of chloroform and light petroleum. A fraction having OMe content of 38.2% was used in subsequent work. It had \([\alpha]_D^{17} -37.0^\circ (c, 0.91 \text{ in } \text{CHCl}_3).\)

4. The specific viscosity of the methylated xylan in solution in m-cresol was determined and the degree of polymerisation of the molecule was calculated to be ca. 66.

5. On hydrolysis the methylated xylan yielded sugars in the following molar ratios: 2:3:4-tri-O-methyl-
D-xylose (1.00), 2:3:5-tri-\(\beta\)-methyl-L-arabinose (0.79), 2:3-di-\(\beta\)-methyl-D-xylose (4.00), 2-\(\beta\)-methyl-D-xylose (1.14), and a methylated acid fraction identified as 3-\(\beta\)-methyl-2-\(\beta\)-(2:3:4-tri-\(\beta\)-methyl-\(\alpha\)-D-glucopyranuronosyl)-D-xylose. The molar ratios were calculated, for the neutral sugars, from the weights of the fractions recovered from a cellulose column. The fraction weights were corrected for impurity.

6. A structure is suggested for the xylan on the basis of the above results and the structure is supported by periodate oxidation studies on the xylan.

An Oat Straw Arabinose-rich Hemicellulose.

1. The hemicelluloses extracted with alkali from oat straw holocellulose contained 5.6% of arabinose residues.

2. After fractionation of the copper complexes of the hemicelluloses the xylan regenerated contained 3.0% of arabinose residues. Further fractionation did not reduce this percentage. It was deduced that the crude hemicelluloses contained at least two hemicelluloses and that in one of these the ratio of arabinose to xylose residues was at least 1 to 5.
3. Attempts to isolate an arabinose-rich hemicellulose from the crude hemicellulosic material by fractional extraction and by other techniques failed to yield any product with a ratio of arabinose to xylose residues higher than 1 to 10.

The Enzymic Hydrolysis of Hemicelluloses.

1. The xylanase and other enzymic activities of five enzyme preparations were investigated.

2. A commercial taka-diastase investigated had a low xylanase activity. It catalysed the hydrolysis of oat straw xylan giving, inter alia, as end product a polysaccharide which contained xylose and arabinose residues (10:1).

3. Enzyme preparations from Cladophora rupestris catalysed the hydrolysis of oat straw xylan to give as end products xylose and arabinose whilst they catalysed the hydrolysis of esparto grass araboxylan giving, as end product, xylobiose but neither xylose or arabinose.

4. A commercial enzyme preparation named "Hemicellulase" was investigated. It possessed xylanase, mannanase, glucosanase, amylase, sucrase, cellulase, and inulinase activities. It did not appear to have any trans-xylosylase activity.
5. The optimum pH for the action of the xylanase(s) was 4.

6. An enzyme possessing cellulase, but no xylanase activity and an enzyme possessing xylanase but no cellulase activity were detected on ionophoretograms.

7. The xylanase activity of the "Hemicellulase" was attributed to at least two enzymes one of which catalysed the hydrolysis of the xylan giving, as principal end-product, xylobiose whilst the other enzyme possessed xylobiase activity.

8. The xylobiase activity of the "Hemicellulase" was destroyed by incubating a digest containing "Hemicellulase" and papain.

9. The "Hemicellulase" appeared to attack beechwood xylan and esparto grass araboxylan in a similar way to oat straw xylan.

10. Attempts were made to modify the xylanase activities of the "Hemicellulase".

11. The "Hemicellulase" has been fractionated by chromatographic techniques.
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