262. *The Constitution of Xylan from Esparto Grass* (Stipa tenacissima, *L.*).

By S. K. Chanda, E. L. Hirst, J. K. N. Jones, and E. G. V. Percival.

A xylan containing no arabinose residues has been prepared from esparto grass. After the hydrolysis of its methylated derivative and the chromatographic separations of the products, both on paper strips and on a column of cellulose, it is concluded that the polysaccharide consists of a singly branched molecule containing 75 ($\pm$5) D-xylopyranose units, the single branching point being formed by a 1 : 3-union. This conclusion is supported by the results of oxidation with periodate, and by estimation of the reducing power with 3 : 5-dinitrosalicylic acid and of molecular weight by the osmotic-pressure method.

The chemistry of the formation and inter-relations of the various components of plant cell-wall materials is complex, and the theories put forward in explanation are not always backed by rigid experimental evidence. These materials are probably some of the end products of the metabolism of the cell protoplasm, but the biochemistry is not as yet sufficiently well understood to enable one to follow stage by stage the process of the formation of the cell-wall materials. In general terms, the cell-wall carbohydrate materials can be divided into three classes—" pectin " which is removed by neutral or acid extraction, " hemicelluloses " which are removed from the remainder by various strengths of alkali, and the residual " cellulose." In the woody tissues of higher plants these substances are generally associated with lignin. Simple examination shows that each fraction is a complex mixture, and a critical examination of each of the individual polysaccharides is necessary, therefore, before one can obtain an exact picture of the amount and distribution of the total polysaccharides. In the present work we discuss the chemistry of xylan which is one of the main components of the hemicellulose group.

It has been known for some time that combined arabinose (6%) is associated with xylan. The early experiments appeared to indicate that the arabinose was present as an end group in a molecule comprising 18—20 xylopyranose residues (Haworth, Hirst, and Oliver, *J.*, 1934, 1917), occurring in the furanose form and, therefore, very susceptible to hydrolysis. It could be eliminated by hydrolysis with 0·005N-oxalic acid without seriously affecting the chain length (Bywater, Haworth, Hirst, and Peat, *J.*, 1937, 1983). It was found that fractional precipitation as the copper complex progressively reduced the total arabinose content of xylan, suggesting that the so-called " araboxylan " or " xyloaraban " might be, not a homogeneous polysaccharide, but a mixture of a true xylan with an araban of the type present in pectic materials.

The xylan was isolated from esparto holocellulose by extraction with dilute sodium hydroxide solution at room temperature, followed by acidification with acetic acid and precipitation with

acetone. After reprecipitation, a white product was obtained, similar in composition to the esparto xylans previously examined (Haworth, Hirst, and Oliver, *loc. cit.*; Bywater, Haworth, Hirst, and Peat, *loc. cit.*) in respect of the xylose (84%) and arabinose (7·5%) contents, although glucose (5·7%) was also found in the products of hydrolysis. These constituents were determined by quantitative paper chromatography (Flood, Hirst, and Jones, *J.*, 1948, 1679).

After several precipitations, as the copper complex, with Fehling's solution, followed by extraction with water to remove a soluble polyglucosan, a xylan was obtained which on hydrolysis gave neither glucose nor arabinose. The xylan, $[\alpha]_D$ −92° (*c*, 0·5 in 0·5N-sodium hydroxide), contained 0·5% of ash and 0·3% of lignin, and on hydrolysis gave D-xylose in 98% yield calculated by determination as furfuraldehyde phloroglucide or thiobarbiturate and in 95% yield as the crystalline dibenzylidene dimethyl acetal (Breddy and Jones, *J.*, 1945, 738). By filter-paper chromatography (Flood, Hirst, and Jones, *loc. cit.*) and determination by the Somogyi reagent, 97—98% of the xylan was accounted for as D-xylose, and no other sugars could be detected. A control experiment on the hydrolysis of β-methylxyloside under the experimental conditions used for the hydrolysis of the polysaccharide showed a loss of xylose amounting to 2%, so that the above results indicate a quantitative yield. Uronic acids were proved to be absent both by paper-chromatographic examination and by the naphtharesorcin test. Furthermore the yield (0·4%) of carbon dioxide obtained on heating the xylan with hydrochloric acid (19%) was comparable with that given by xylose itself under the same conditions.

The extraction of xylan and its delignification were carried out under mild conditions in order to minimise the possibility of degradation. During the purification by means of the copper complex no obvious signs of degradation were noted; in particular the value for the specific viscosity [$\eta_{sp.}/c$ = 10 in sodium hydroxide (0·5N.)] remained constant throughout the various stages.

By two series of five methylations, each with methyl sulphate and sodium hydroxide in an atmosphere of nitrogen, a methylated xylan was prepared, having $[\alpha]_D$ −85° (in chloroform) and containing 38% of methoxyl (Calc. for $C_7H_{14}O_4$: OMe, 38·7%). This was divided by fractionation using chloroform–light petroleum into two main fractions: (*A*) soluble in 3 : 7 chloroform–light petroleum and having OMe 38·6%, and (*B*) soluble in 3·5 : 6·5 chloroform–light petroleum and having OMe, 36·7%. On further methylation with methyl iodide and silver oxide (*B*) gave a product similar in properties to (*A*), including viscosity in *m*-cresol.

Paper-chromatographic examination (Hirst, Hough, and Jones, *J.*, 1949, 928) of the hydrolysates of the fully methylated samples described above indicated the presence of (*a*) 2 : 3 : 4-trimethyl xylose, (*b*) 2 : 3-dimethyl xylose, (*c*) monomethyl xylose, and (*d*) a trace of xylose. The sugars were identified by measuring the R_G values, confirmation being obtained by running standard sugars against the hydrolysate. The proportions of the various sugars in the hydrolysate were determined by hypoiodite oxidation after separation on a paper chromatogram, and were found to be (*a*) trimethyl xylopyranose 2·4—2·8% (molar) and (*c*) monomethyl xylose, 4·5—5·5%. The oxidation was carried out in sodium hydroxide–phosphate buffer (pH 11·4) (Ingles and Israel, *J.*, 1948, 810) and not in carbonate-bicarbonate buffer (pH 10·6), thereby removing the possibility of losing iodine through effervescence during the acidification of the solution. The reagent was found to oxidise stoicheiometrically the common aldoses and their methylated derivatives, in particular those of xylose. The chain length calculated on this result indicated one non-reducing end group per 36—41 residues.

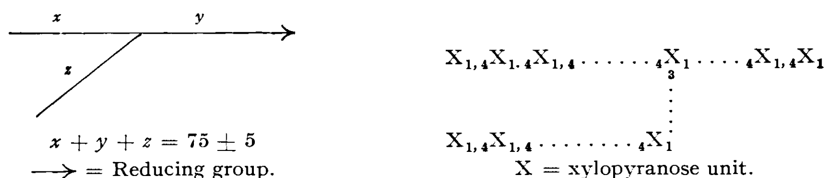
A similar result was also obtained when a quantity of methylated xylan hydrolysate was separated on a cellulose column (Hough, Jones, and Wadman, *J.*, 1949, 2511). The methylated xylan (6·2 g.) was converted first into the methylglycosides by 1% methanolic hydrogen chloride ($[\alpha]_D$ +71°, constant after 8 hours) and then into the free sugars by 0·5N-hydrochloric acid ($[\alpha]_D$ +55°, changing to +24° in 6 hours). The hydrolysate was concentrated to a syrup in each case and placed on the top of the column. It was developed with *n*-butanol–light petroleum (b. p. 100—120°) (3 : 7) saturated with water and containing 1% of ammonia, until the fully methylated pentose and 2 : 3-dimethyl xylose were removed. Monomethyl xylose did not travel sufficiently rapidly in this solvent, and the developing solution was therefore changed to *n*-butanol containing some water. An examination of the different fractions of the eluate showed a perfect separation of the sugars. The amount of pure 2 : 3 : 4-trimethyl xylose was 200 (±15) mg. The 2 : 3-dimethyl xylose weighed 6 g. and the amount of 2-methyl xylopyranose was 250 mg. Values calculated from the results of hypoiodite oxidations and determinations of specific rotation and methoxyl content were in agreement with these weights. Some 95% of the polysaccharide investigated was accounted for, a result much better than

could be expected by fractional distillation of the glycosides, a method inherently defective on account of losses by pyrolysis and incomplete separation. It was also proved that the yields both of the end group and of the partly methylated sugars could not be seriously affected by losses caused by demethylation during acid hydrolysis. A similar experiment carried out on chromatographically pure samples of 2 : 3 : 4-trimethyl and 2 : 3-dimethyl xylose showed (paper chromatography) that the former gave at most 1·5% of dimethyl and 0·4% of monomethyl xylose, whilst the latter gave only 0·3% of monomethyl derivative.

The trimethyl and the monomethyl xylose were both crystalline, and were identified as 2 : 3 : 4-trimethyl D-xylopyranose and 2-methyl D-xylopyranose respectively, confirmation being obtained by forming the anilides. *X*-Ray crystallographic examination (by courtesy of Dr. C. A. Beevers, Dewar Crystallographic Laboratory, Edinburgh University) of the trimethyl sugar showed it to be identical with trimethyl xylopyranose. The 2 : 3-dimethyl D-xylose was identified as its anilide, and as the lactone, amide, and *p*-bromophenylhydrazide of the corresponding 2 : 3-dimethyl xylonic acid.

The amount of trimethyl xylose obtained by quantitative separation of the methylated xylan hydrolysate on the cellulose column corresponded to 35 ± 3 residues per non-reducing end group. The molecular-weight determination of this sample by osmotic-pressure measurement (by courtesy of Professor H. W. Melville, F.R.S., of the University of Birmingham) gave a value of 11,000 (D.P. 70). Results of the same order were also obtained from the viscosities of the methylated (D.P. 86—90) and the acetylated xylan (D.P. 83). The viscosity in *m*-cresol was determined in an Ostwald viscometer, and molecular weights were calculated using the values $K_m = 12 \times 10^{-4}$ in the case of methylated xylan, and $K_m = 6{\cdot}3 \times 10^{-4}$ for the acetylated derivative. These constants are those valid for the corresponding cellulose derivatives, and it is realised that they may not be strictly applicable to a pentose polymer, particularly one for which it seems necessary to propose a branched structure. Nevertheless, the calculation of the molecular weights using these constants gave values closely similar to those obtained by osmotic-pressure measurement. The small discrepancy may be due to the branched structure, which, since only one branch point is involved may not seriously affect the results.

It is probable from these results that the xylan molecule comprises 70—80 β-D-xylopyranose units linked by 1 : 4-linkages (Haworth and Percival, *J.*, 1931, 2850) and terminated by one reducing and two non-reducing groups. The proportion of reducing groups in the xylan molecule will be discussed presently. On the above basis the xylan molecule will consist of a single branched chain which may be represented as follows :



It follows that the fully methylated polysaccharide on hydrolysis should give one mole of monomethyl xylose per 2 moles of the end group. Just over a molar proportion of 2-methyl xylose was in fact separated and identified in the hydrolysate, indicating that the branching occurred through the position $C_{(3)}$ of a xylose residue in the 1 : 4-linked chain. The excess over the theoretical yield of the aforesaid monomethyl compound might well have arisen by demethylation of the 2 : 3-dimethyl xylose; the possibility of incomplete methylation in the first case, although improbable, cannot be excluded entirely.

This structure is in agreement with the results obtained (*a*) by determining the formic acid liberated from the end groups by periodate oxidation, and (*b*) by determination of the reducing group (several methods). One mole of formic acid will be liberated by periodate from each non-reducing terminal group in the molecule and 2 moles from the reducing end. In practice the amount of formic acid liberated was almost constant after 163 hours and corresponded to one mole per 20—21 residues, indicating 60 residues in an assumed straight chain or 80 on the branched structure proposed above. Very little xylose could be detected on hydrolysis of the fully oxidised xylan. This small residual amount of xylose probably came from the residue at the branching point in the xylan molecule which cannot be oxidised by the periodate. Just over one mole of periodate was necessary to oxidise each $C_5H_8O_4$ residue, in harmony with the presence of 1 : 4-linkages in the chains.

Estimation of the reducing group by colorimetric measurement of the reduction of 3 : 5-dinitrosalicylic acid to nitroaminosalicylic acid by the method of Meyer *et al.* (*Helv. Chim. Acta*, 1948, **31**, 103) gave a value of the order of one reducing group per 70—80 xylose residues. These authors used the absorption curve for maltose instead of glucose in their estimations of the chain length of amylose using the equation : D.P. = 2 × wt. of polyglucose/wt. of maltose. No corresponding disaccharide of xylose is known, but it was apparent from a comparison of the absorption curves for glucose and maltose that the chain length calculated from the latter is higher (nearly 30% higher when the colorimetric reading is not too low) than that obtained by using glucose for reference. As a first approximation, therefore, it may be assumed that a similar correction to the chain length of xylan will be necessary when it is calculated from the xylose curve, and we get, therefore, a value of 78—82 residues. A study of the nature of the three absorption curves for glucose, maltose, and xylose suggested that the absorption value of the hypothetical xylose disaccharide will approximate to that of glucose, and the chain length calculated on that assumption gives a value of 72—79 residues. It is difficult to make a definite decision on this point, but it is likely that the method gives an indication of the right order for the molecular weight.

Hypoiodite oxidation of the polysaccharide in a buffer medium (pH 10·6) indicated one reducing group per 48—50 residues. It is known that some over-oxidation occurs with this reagent, as is also apparent from the results of oxidation in the unbuffered medium, and it is very difficult to control the speed of the reaction. This method does not, therefore, give an accurate figure for the molecular size of the xylan, but it does show that the number of the residues per reducing group in the complex is not less than 50.

From the above considerations it seems justifiable to assign to the xylan from esparto grass a structure containing about 75 (±5) D-xylopyranose units joined by 1 : 4-β-linkages with a single branching point formed by a 1 : 3-union at some point as yet undetermined along the chain. A single-chain molecule is unacceptable in view of the evidence provided by the molecular weight, by osmotic pressure and by end-group determinations.

Experimental.

Preparation of Xylan from Esparto Grass.—The esparto grass [moisture, 9·3; ash, 3·6; lignin, 20·5 (Mahood and Cable, *Ind. Eng. Chem.*, 1922, **14**, 933); pentosan (as phloroglucide), 25·8; uronic anhydride, 3·1%], cut into pieces 3—4 cm. long, was extracted with benzene and methanol, to remove respectively the waxy and colouring materials, and milled to obtain a fibrous product, any powder being rejected. The material contained a high percentage of lignin (21—23%) and was delignified by Wise's method (*Ind. Eng. Chem., Anal.*, 1945, **17**, 63), slightly modified to suit the special conditions. The grass (100 g.) was suspended in water (5 l.) containing acetic acid (500 ml.) and sodium chlorite (500 g.). The solution was heated to 60° with occasional shaking and kept at that temperature for 1 minute. Sodium acetate (20 g.) was then added and the flask was transferred to a bath (thermostat) at 30° and kept at that temperature with occasional shaking for 24 hours. The pH of the solution (4·0) remained constant throughout the reaction. The mixture was filtered through cloth, and the fibrous residues were washed with ice-cold water, to remove the acid, and then with acetone. The product was dried in the air, a white fibrous holocellulose (moisture, 3·5; ash, 1·9; lignin, 2·4; pentosans, 30·2; uronic anhydride, 3·4%) being obtained (70—75 g.). The small amount of lignin did not, however, interfere with the xylan preparation since it could be left almost completely in the acetone solution during the precipitation of the xylan (see below). A second treatment by sodium chlorite removed the lignin completely but there was considerable degradation of the less resistant carbohydrate material.

The xylan was extracted from the holocellulose (100 g.) by rolling it for 24 hours in a " Kilner " jar containing glass marbles and sodium hydroxide solution (2 l.; 4%), the slurry was centrifuged and the crude xylan precipitated with acetone after acidification with acetic acid. This operation was repeated on the residual solid. The small amount of lignin dissolved in the alkali was left in the acetone solution, as indicated by the brown colour. The crude xylan was collected on the centrifuge, and the precipitate was washed with acetone–water (1 : 1) to remove the acid, followed by alcohol of increasing concentration, and finally with ether. The solid was dried in the air till most of the ether had evaporated, whereafter the xylan (27—28 g.) could be powdered and then dried over phosphoric oxide in a vacuum desiccator. The product, $[\alpha]_D$ −91° (*c*, 0·5 in 0·5N-sodium hydroxide) was light brown but, after one or two reprecipitations, became colourless; this was xylan I (moisture, 1·2; ash, 0·58; lignin, 0·3; pentosans, 96·0; uronic anhydride, 0·4%). Chromatographic examination (Flood, Hirst, and Jones, *loc. cit.*) of the hydrolysate showed that the polysaccharide contained arabinose (7·5%) and glucose (5·65%) along with xylose (84%).

Purification of Xylan.—The crude xylan (20 g.) was dissolved in sodium hydroxide (1·5 l.; 4%), and the solution treated with equal volumes of freshly prepared Fehling's solution whereupon the copper complex was precipitated. A little acetone was added for quick settling of the precipitate. The top, clear liquid was decanted off, and the solution filtered through mercerised muslin. The precipitate was suspended in water (1 l.) by vigorous stirring. Cold hydrochloric acid (2N.) was then added carefully to decompose the copper complex, a clear or occasionally slightly opalescent polysaccharide solution being obtained. The acidity of the solution was never allowed to exceed N. The polysaccharide was precipitated with acetone, avoiding excess, to give a white flocculent precipitate.

The solution was centrifuged and the precipitate washed with slightly acidified acetone–water (60 : 40), to remove the copper completely, and then with the solvent mixture to remove the acid, and finally with alcohol and ether. It was dried over phosphoric oxide in a desiccator.

The polysaccharide was then suspended in water (500 c.c.) and shaken overnight to separate the water-soluble fraction. The insoluble fraction obtained on centrifuging was dried as before (xylan II). The glucose and arabinose in the insoluble fraction were progressively removed by such purifications. Usually 2—3 purifications as the copper complex were necessary to obtain from old specimens of esparto grass a xylan free from glucose and arabinose (yield, 80—85%). Some 5—6 such purifications were necessary to obtain pure xylan (xylan III) from a green and relatively fresh sample of the grass (yield, 60—70%). An attempt to fractionate the pure xylan by precipitation with glacial acetic acid was unsuccessful as the polysaccharide was completely precipitated by the acid. The polysaccharide, however, did not seem to be degraded since the same value for the viscosity in N-sodium hydroxide was obtained for xylan at different stages of purification ($\eta_{sp.}/c = 10{\cdot}0$, where c is in Staudinger units). Xylan III had $[\alpha]_D^{21}$ $-92°$ (c, 0·5 in 0·5N-sodium hydroxide) (ash, 0·5; lignin, 0·3; pentosan, 98%). It gave 95% of the theoretical amount of xylose after hydrolysis [estimated as xylose dibenzylidene dimethyl acetal by the Breddy–Jones reagent (*loc. cit.*), using the equation $y = 0{\cdot}5175x + 0{\cdot}0457$ where y is the weight of xylose and x the weight of the derivative]. Filter-paper chromatographic estimation of the xylose (no other sugar could be detected) obtained on hydrolysis with 0·5N-sulphuric acid, with Somogyi's copper reagent, accounted for 97—98% of xylan. A control experiment of the hydrolysis of β-methylxyloside under identical conditions indicated that nearly 2% of the xylose was destroyed during hydrolysis. The polysaccharide, therefore, consisted of xylose only and was obtained in a very pure state (98—99%). No uronic acid could be detected in the pure polysaccharide either by paper chromatogram or by the naphtharesorcinol test.

Methylation of Xylan.—Xylan III (18 g.) was suspended in water (100 c.c.) in a three-necked round-bottomed flask and allowed to swell overnight. The air was then displaced by nitrogen, a gentle stream of the gas being maintained throughout. Sodium hydroxide [200 c.c.; 40% (by wt. throughout)] was then added with vigorous mechanical stirring, and the solution was stirred for 4 hours. Methyl sulphate (180 c.c.) was then added dropwise during 6—8 hours, the flask being cooled in ice and water. After overnight stirring the reaction was completed by heating in a water-bath for 1 hour. The mixture was cooled and treated with sodium hydroxide (300 c.c.; 40%) followed by methyl sulphate (180 c.c.) as before, and this process was repeated 4 times at room temperature in nitrogen; cooling in ice was unnecessary after the first methylation. Before the fifth methylation acetone (200 c.c.) was added; it was removed by distillation on completion of the methylation. The mixture was then cooled and treated with sulphuric acid (0·5N.) until it was faintly alkaline (pH 8); it was then boiled with water, whereupon the precipitated sodium sulphate dissolved and the methylated xylan separated. The product was filtered hot through cloth, washed with hot water, and dried at 95°/15 mm. (Found: OMe, 32·2%). The aqueous solution and aqueous washings were extracted with chloroform, the solvent was removed, and the product added to the main bulk. The partly methylated xylan was dissolved in aqueous acetone (80%), and then treated with sodium hydroxide (300 c.c.; 40%) for 2 hours with stirring under nitrogen, followed by methyl sulphate as before. A second series of five methylations was carried out to give methylated xylan (20·2 g.), $[\alpha]_D^{20}$ $-83°$ (c, 0·8 in chloroform) (Found: OMe, 36·4%).

Fractionation of Methylated Xylan.—The methylated xylan (20 g.) was treated with 300 c.c. (in two batches of equal volume) of purified light petroleum (b. p. 60—65°)–chloroform, the amount of the latter solvent in the mixture being increased in stages. For each extraction the mixture was boiled gently in a water-bath for 2 hours, the insoluble material allowed to settle, and the clear liquid decanted. The solvent was then removed under diminished pressure and the residue dried at 90—95°/15 mm. over phosphoric oxide to constant weight. The results are collected in Table I.

TABLE I.

Fraction.	Solvent, $CHCl_3$–light petroleum.	Yield, %.	Sulphated ash, %.	OMe, %.	$[\alpha]_D^{20}$.
1	0 : 100	0·6	Nil	1·5	−3°
2	10 : 90	0·9	0·04	4·2	−7
3	15 : 85	0·25	0·09	18·5	−26·5
4	20 : 80	0·4	0·11	22·0	−33
5	25 : 75	0·4	0·11	38·9	−83
6	30 : 70	41·3	0·2	37·9	−85
7	35 : 65	37	1·4	36·7	−84·5
8	Residue	15·9	6·2	34·8	—

Fraction 7 was treated with methyl iodide (200 c.c.) and silver oxide (100 g.), and after the usual treatment had OMe, 38%. The fraction was then completely soluble in 3 : 7 chloroform–light petroleum. Fractionation was carried out as before (Table II).

TABLE II.

Fraction.	Solvent, $CHCl_3$–light petroleum.	Yield, %.	Sulphated ash, %.	OMe, %.	$[\alpha]_D^{20}$.
7A	0 : 100	0·4	—	Nil	Nil
7B	15 : 85	0·3	Nil	25·0	−42°
7C	20 : 80	0·15	—	37·5	−81
7D	25 : 75	7·3	0·14	38·4	−85
7E	30 : 70	91·6	0·37	38·6	−85·5

Measurements of viscosity (see below) and other analytical data showed fractions 6 and 7E to be identical (Found : C, 52·5; H, 7·7. Calc. for $C_7H_{12}O_4$: C, 52·5; H, 7·5%).

Acetylation of Xylan.—Xylan (3·5 g.) was warmed with pyridine (150 c.c.) at 70° for 2 hours. After the mixture had been cooled to 15° acetic anhydride (40 c.c.) was added dropwise with shaking and the flask kept in the dark for 3 days with occasional stirring. The contents were then poured into water and the insoluble product was separated at the centrifuge, washed with water, alcohol, and ether and dried at room temperature over phosphoric oxide (Found : CH_3·CO, 38·7%. Calc. for $C_9H_{12}O_6$: CH_3·CO, 39·8%).

A second acetylation of this material gave a product (5·4 g.) with the theoretical acetyl content. The xylan acetate was insoluble in methanol, ethanol, acetone, or chloroform; it was soluble to some extent in pyridine and chloroform–ethanol (9 : 1) and had $[\alpha]_D^{16}$ −155° (*c*, 1·0 in *m*-cresol).

Viscosity Determination of Xylan and its Derivatives.—The viscosities of xylan in 0·5N-sodium hydroxide and its derivatives in *m*-cresol were measured in an Ostwald viscometer at 20° (Table III).

TABLE III.

Derivative.	Sample.	*c*.	Average time of flow in secs., solution.	solvent.	$\eta_{sp.}/c$.	*M*.	D.P.
Xylan	Xylan I	0·1272	671·5	293	10·16	—	—
	Xylan II	0·1246	657	,,	10·02	—	—
	Xylan III	0·1363	684	,,	9·8	—	—
Acetylated xylan	Insoluble fraction	0·046	760	501	11·24	17,840	83
Methylated xylan	6	0·126	1541	,,	16·47	13,720	86
	7E	0·1356	1694	,,	17·56	14,630	91
	5	0·1323	1096	,,	8·97	7,474	47
	7D	0·1414	1111	,,	8·61	7,174	45

Molecular weights were calculated for the acetyl and methyl derivatives from the equation $\eta_{sp.} = K_m Mc$, were *c* is concentration in g.-mol. of repeating units per litre. K_m for acetate 6·3 × 10^{-4} and K_m for the methylated xylan 12 × 10^{-4} (*i.e.*, the constants applied to the corresponding cellulose derivatives; Staudinger and Reinecke, *Annalen*, 1938, **535**, 47).

A determination, carried out through the kindness of Professor H. W. Melville, F.R.S., of the molecular weight of methylated xylan (6) gave a value of 11,000 (D.P. 70).

Hydrolysis of Methylated Xylan and Separation of Methylated Xyloses.

(a) *By Paper Chromatography.*—The polysaccharide (100 mg.) was treated with methanolic hydrogen chloride (5 c.c.; 1%) in a sealed tube at 100° for 7—8 hours. After careful removal of the solvent, the residual syrup was hydrolysed for 6—8 hours with hydrochloric acid (10 c.c.; 0·5N.), neutralised with silver carbonate, and filtered, and silver was then removed from the filtrate by hydrogen sulphide and basic or acidic ions by " Deacidite B " and " Zeocarb H1 ". The clear solution was concentrated to a thin syrup at 35°/15 mm. Examination on the paper chromatogram with butanol–ethanol–water showed, on development with aniline oxalate, three distinct spots corresponding to a trimethyl pentose (R_G 0·94—0·95), 2 : 3-dimethyl xylose (R_G 0·74—0·76), and monomethyl xylose (R_G 0·38—0·41), together with a trace of xylose.

The method of Hirst, Hough, and Jones (*J.*, 1949, 928) for the estimation of methylated sugars by alkaline hypoiodite after separation on the paper chromatogram was modified by the use of a sodium hydroxide–phosphate buffer (pH 11·40) (Ingles and Israel, *J.*, 1948, 810), the method being shown (Table IV) to be applicable to the methylated sugars concerned. A known sugar solution (5 c.c.) was treated with iodine (1 c.c.; 0·1N.) measured from an " Agla " micrometer syringe (in two batches), followed by 2 c.c. of the buffer (25 c.c. of 0·1N-disodium hydrogen phosphate and 8·67 c.c. of 0·1N-sodium hydroxide, diluted to 50 c.c.) into a " Quickfit " boiling tube, which was then quickly closed with a stopper moistened with 10% potassium iodide solution. The tube was kept in a cool, dark place for 6 hours, the stopper washed with water, and the solution acidified with sulphuric acid (2 c.c.; 2N.) and titrated with sodium thiosulphate (0·01N.). A blank was run concurrently.

TABLE IV.

Sample.	Weight, mg.	Weight, found, mg.	Recovery, %.	Sample.	Weight, mg.	Weight, found, mg.	Recovery, %.
2 : 3 : 4-Trimethyl xylose	0·215	0·213	99·1	Xylose	0·501	0·496	99·0
	0·430	0·426	99·1		1·002	0·997	99·5
	1·073	1·080	100·6		2·506	2·428	97·0
	2·146	1·990	92·7				
				Ribose	0·612	0·604	98·7
2 : 3-Dimethyl xylose	0·395	0·378	95·7		1·224	1·214	99·2
	0·988	0·966	97·8		3·060	2·907	95·0
	1·977	1·898	96·0				
				Rhamnose (hydrate)	0·559	0·494	88·4
2-Methyl xylose	1·230	1·214	98·7		1·118	0·961	86·0
	2·310	2·267	99·0		2·236	1·491	66·6
					2·795	1·689	60·4

It will be observed that, with the exception of rhamnose, the oxidation is practically stoicheiometric at low concentrations of sugars.

Methylated xylan (50 mg.) was hydrolysed as described above. Heavy spotting was necessary to determine the end group and a wide paper (21 cm.) was used, and the blank paper cut from the same sheet was hung from the opposite side of the trough. The filter-paper strips containing the sugars were extracted for 45 minutes with 5 c.c. of water contained in a boiling tube, cooled to room temperature, and analysed by the above method (see Table V). For the estimation of the dimethyl xylose the quantities of reagents was increased tenfold.

TABLE V.

Sugar.	Wt., mg.	Molar composition, %.	Sugar.	Wt., mg.	Molar composition, %.
Sample 6.			*Sample* 7E.		
2 : 3 : 4-Trimethyl xylose	0·26, 0·28	2·5, 2·8	2 : 3 : 4-Trimethyl xylose	0·20, 0·19	2·4, 2·7
2 : 3-Dimethyl xylose	8·64, 8·57	91·5, 91·7	2 : 3-Dimethyl xylose	7·10, 5·97	92·1, 91·5
2-Methyl xylose	0·49, 0·44	5·5, 5·0	2-Methyl xylose	0·32, 0·28	4·5, 4·7
Xylose	0·03, 0·03	0·4, 0·3	Xylose	0·06, 0·06	0·9, 1·0

These results indicate one reducing group per 36—41 xylose units.

(b) *By the Cellulose Column.*—Methylated xylan (6·2 g.) was boiled gently on a water-bath with methanolic hydrogen chloride (620 c.c.; 1%), the following rotations being observed: $[\alpha]_D^{16}$ +4° (1 hr.), 14° (2 hrs.), 20° (3 hrs.), 35° (4 hrs.), 51° (5 hrs.), 60° (6 hrs.), 68° (7 hrs.), 71° (8 hrs., constant). The solution was then cooled in ice-salt, and the acid neutralised by the addition of a dry ethereal solution of diazomethane. The solution was then concentrated to a syrup, which was dissolved in ether, filtered, concentrated, and heated at 40°/15 mm., to give a syrup (7·43 g.) which was hydrolysed at 95—100° with hydrochloric acid (310 c.c.; 0·5N.) and then had $[\alpha]_D^{16}$ +55° ⟶ +24·2° (6 hours, constant). The acid solution was neutralised with silver carbonate (previously triturated with water) and filtered, and silver was then removed with hydrogen sulphide. The solution was filtered through washed charcoal and concentrated to a syrup (7·0 g.) at 40°/15 mm.

A column of powdered cellulose (60 × 4 cm.) was prepared, washed, and tested as described by Hough, Jones, and Wadman (*J.*, 1949, 2511). The solvent selected for the operation after a preliminary trial with butanol–water was light petroleum (b. p. 100—120°)-*n*-butanol (7 : 3), saturated with water and containing 1% of ammonia. The above syrup was then dissolved in the minimum volume of the solvent for dissolution, added dropwise to the top of the column and allowed to soak in. A thin layer of cotton wool was then placed on the top of the column, followed by 100 c.c. of the solvent; the column was then left overnight. The reservoir was filled with solvent and the column developed by using the automatic device for changing the receiver every 5 minutes. 1 C.c. of the eluate from every tenth tube was concentrated on a watch-glass over a water-bath, and small spots were placed in chronological order along the starting line of filter-paper chromatogram and separated in the usual way. The residues on the watch-glasses were dissolved in acetone and transferred quantitatively to the respective tubes. On development of the paper a picture of the distribution of the sugars was obtained. Trimethyl xylose was completely separated between tubes 50 and 70, after a gap of 20 tubes dimethyl xylose appeared (90—240), and no sugars were eluted between tubes 240 and 500. At the 500th tube the solvent was changed to *n*-butanol, monomethyl xylose being collected between tubes 650 and 710. The column was then washed with water to obtain the trace of xylose known to be present and any residue remaining at the top of the column. The tubes were then grouped, the solvent was removed at 35°/15 mm., and the residue dissolved in water and filtered through charcoal to remove waxy impurities. After further concentration and drying by the addition of methanol and distillation, the following fractions were obtained: (1) trimethyl pentose (0·272 g.); (2) dimethyl pentose (6·03 g.); (3) monomethyl pentose (0·265 g.); and (4) xylose (0·06 g.).

Examination of the fractions. Fraction (1). Partial crystallisation took place gradually. The crystals (109 mg.) were separated on a tile and washed (53·6 mg.), the tile was extracted with chloroform, and the extract and washings were evaporated. A small quantity of material insoluble in methanol was removed, and two further crops of crystals (44·4 mg.) were removed. The residual syrup (136·3 mg.) was twice purified from light petroleum containing a trace of butanol, and a small insoluble residue rejected. The syrup eventually obtained (110 mg.) crystallised completely in one week (purity by hypoiodite oxidation 93—94%) and had $[\alpha]_{15}^{D}$ +20·3° (*c*, 1·1 in water), m. p. 87° raised to 90° on recrystallisation from ether (Found: OMe, 46%). The crystals (98 mg.) obtained from the first three batches had m. p. 89—90°, not depressed on admixture with trimethyl D-xylopyranose. Hypoiodite oxidation indicated 100% purity (Found: C, 50·1; H, 8·3; OMe, 48·5. Calc. for $C_8H_{16}O_5$: C, 50·0; H, 8·3; OMe, 48·4%).

Examination by the *X*-ray powder photograph method, by the kindness of Dr. C. A. Beevers, showed the substance to be 2 : 3 : 4-trimethyl xylose. The derived anilide had m. p. 102° alone or in admixture with an authentic specimen (Laidlaw and Percival, *J.*, 1949, 1600) (Found: C, 62·7; H, 7·8; N, 5·1; OMe, 34·9. Calc. for $C_{14}H_{21}O_4N$: C, 62·9; H, 7·8; N, 5·2; OMe, 34·8%).

The total quantity of trimethyl D-xylopyranose obtained was 210 (±15) mg., corresponding to one end group in 35 ± 3 residues.

The small quantity of insoluble residue obtained during the crystallisations was treated with water, and the concentrated filtrate examined chromatographically. No reducing sugars were detected.

Fraction (2). The syrup (6·03 g.) had $[\alpha]_D^{15}$ +23° (*c*, 2·4 in water), n_D^{18} 1·4770 (Found : OMe, 34·9. Calc. for $C_7H_{14}O_5$: OMe, 34·8%). The purity by the hypoiodite method was 98%. The derived lactone had $[\alpha]_D^{15}$ +97°, falling to +62·5° (720 hours, constant; *c*, 1·0 in water), n_D^{19} 1·4640 (Found : OMe, 34·7. Calc. for $C_7H_{12}O_5$: OMe, 35·2%).

The lactone was treated with methanolic ammonia to give 2 : 3-dimethyl D-xylonamide in theoretical yield, m. p. 134° alone or admixed with an authentic specimen, $[\alpha]_D^{14}$ +49° (*c*, 0·8 in water) (Found : C, 43·8; H, 7·9; N, 7·0; OMe, 32·0. Calc. for $C_7H_{15}O_5N$: C, 43·5; H, 7·7; N, 7·25; OMe, 32·1%).

Treatment in ether with *p*-bromophenylhydrazine gave 2 : 3-dimethyl D-xylono-*p*-bromophenylhydrazide (83%), m. p. 150° alone or admixed with an authentic specimen (Found : C, 43·1; H, 5·4; N, 7·75; Br, 21·8; OMe, 16·1. Calc. for $C_{13}H_{19}O_5N_2Br$: C, 43·0; H, 5·3; Br, 22·1; OMe, 17·1%).

Fraction (2) was converted into the anilide, m. p. 145° (alone or admixed with authentic 2 : 3-dimethyl D-xylose anilide), $[\alpha]_D^{14}$ +190° (*c*, 1·2 in ethyl acetate; const. for 24 hours), +118° (5 minutes), +96° (10 minutes), +80° (25 minutes), +75° (45 minutes, constant) [*c*, 0·9 in ethyl acetate containing acetic acid (5% v/v)] (Found : C, 61·6; H, 7·5; N, 5·7; OMe, 24·3. Calc. for $C_{13}H_{19}O_4N$: C, 61·6; H, 7·6; N, 5·5; OMe, 24·5%). Fraction (2) was, therefore, entirely 2 : 3-dimethyl D-xylose.

Fraction (3). The syrup (265 mg.) crystallised almost completely on inoculation with 2-methyl D-xylose. Direct comparison against authentic samples of 2- and 3-methyl xylose on the filter-paper chromatogram showed both crystals and adhering syrup to correspond to the former sugar. Washing with hot methanol gave 2-methyl β-D-xylose (152 mg.), m. p. 134° alone or in admixture with an authentic specimen, $[\alpha]_D^{14}$ +5° (10 minutes), 20° (30 minutes), 30° (2 hours), 34·5° (3 hours, constant) (*c*, 1·0 in water). Hypoiodite oxidation indicated a purity of 97% (Found : C, 43·9; H, 7·4; OMe, 18·5. Calc. for $C_6H_{12}O_5$: C, 43·9; H, 7·3; OMe, 18·9%). The syrup (111 mg.) recovered from the washings had $[\alpha]_D^{15}$ +32·4° (*c*, 1·1 in water) (Found : OMe, 18·3%). Hypoiodite oxidation indicated 96% monomethyl xylose, and the syrup crystallised almost completely when kept for a week, to give 2-methyl xylose, m. p. 134°.

Conversion into the anilide gave a product, m. p. 125° alone or admixed with 2-methyl D-xylose anilide (Found : C, 59·6; H, 7·1; N, 5·8; OMe, 13·1. Calc. for $C_{12}H_{17}O_4N$: C, 60·2; H, 7·1; N, 5·85; OMe, 13·0%).

Fraction (4). The semi-solid syrup (60 mg.) had $[\alpha]_D^{15}$ +12·5° (*c*, 3·0 in water) and was shown by filter-paper chromatography to contain xylose only, the amount calculated from the rotation being *ca.* 40 mg.

The Partial Demethylation of 2 : 3 : 4-Trimethyl and 2 : 3-Dimethyl Xylose.—Specimens of the appropriate sugars (100 mg.) were treated with methanolic hydrogen chloride (20 c.c.; 1%) for 8 hours, followed by aqueous hydrochloric acid (20 c.c.; 0·5N.) for 8—10 hours, under the conditions used for the hydrolysis of methylated xylan. The sugars were isolated in the usual way, dissolved in acetone, filtered to remove inorganic material, concentrated, and dried. The various sugars present were estimated on the paper chromatogram with buffered hypoiodite as described previously; 2—3 larger papers were needed to obtain sufficient of the demethylated sugars for estimation. The results are collected in the following table.

	0·01N-$Na_2S_2O_3$, c.c.	Methylated sugar, %.
2 : 3 : 4-*Trimethyl Xylose.*		
Trimethyl xylose ...	23·83; 25·08	98; 98
Dimethyl xylose ...	0·38; 0·40	1·6; 1·6
Monomethyl xylose	0·09; 0·10	0·4; 0·4

	0·01N-$Na_2S_2O_3$, c.c.	Methylated sugar, %.
2 : 3-*Dimethyl Xylose.*		
Dimethyl xylose ...	45·8; 46·06	99; 99
Monomethyl xylose	0·11; 0·24	0·14; 0·30

Other Experiments.

Periodate Oxidation of Xylan.—(1) *Determination of formic acid released.* The method of Halsall, Hirst, and Jones (*J.*, 1947, 1399, 1427) was used as modified below. Dry xylan (100 mg.) was weighed into each of a number of 50-c.c. stoppered bottles and treated with potassium chloride solution (5 c.c.; 16%), followed by sodium metaperiodate (10 c.c.; 0·083M.) and water (10 c.c.). Blanks were also started. The bottles were shaken continuously in the dark. Bottles were removed at intervals, the contents centrifuged, and 20 c.c. of the clear solution treated with ethylene glycol (1 c.c.) and titrated with sodium hydroxide solution (0·01N.) from a micro-burette (methyl-red). The alkali required for blank experiments (20 c.c.) was small (0·03 c.c.) and did not vary with time. A suspension of the xylan in water was neutral to methyl-red. The following results [moles ($\times 10^2$) of $H \cdot CO_2H$ per $C_5H_{10}O_5$ unit] were obtained : 1 hour, 0·17; 3 hours, 0·65; 73 hours, 4·4; 115 hours, 4·6; 163 hours, 4·8; 190 hours, 4·85; 235 hours, 4·9; 332 hours, 5·1. The amount of formic acid liberated was practically constant after 163 hours, corresponding to 1 mole per 20—21 xylose residues.

(2) *Uptake of periodate.* Batches (100 mg.) of xylan were shaken in the dark with sodium metaperiodate solution (25 c.c.; 0·25M.), the rotation becoming strongly positive. Samples were taken at intervals of 24 hours and diluted to 250 c.c. The amount of periodate consumed (per $C_5H_8O_4$ unit) was determined (Fleury and Lange, *J. Pharm. Chim.*, 1933, 8, **17**, 107, 196) : 0·96 (24 hours); 1·15 (48 hours); 1·28 (72 hours). The completely oxidised solution in a Cellophane bag was then dialysed against tap water until free from inorganic ions, concentrated at 35°/15 mm. to 1—2 c.c., and hydrolysed at 100° in a sealed tube with concentrated sulphuric acid (0·04 c.c.). After neutralisation with barium carbonate, examination on the filter-paper chromatogram indicated the presence of a trace of xylose.

The Reducing Power of Xylan.—(1) *Hypoiodite oxidation.* The polysaccharide (100 mg.) in water (10 c.c.) was treated with sodium hydroxide (10 c.c.; 2N.), followed by iodine (10 c.c.; 0·1N.). After two batches had been kept in the dark for 3 hours and 16 hours respectively, the solutions were acidified with sulphuric acid (25 c.c.; 2N.), and the regenerated iodine was titrated with sodium thiosulphate. Blanks were run concurrently. The results corresponded to one reducing group per 43—47 xylose residues. In another experiment the oxidation was conducted as before (3 hours) with the equivalent

of 40 c.c. of 0·1N-sodium hydroxide and 20 c.c. of 0·1N-iodine buffered with sodium carbonate (0·2M.)–sodium hydrogen carbonate (0·2M.) solution (5 c.c.). Excess of iodine was titrated after acidification, care being taken to lose no iodine during effervescence, giving a value of one reducing group per 48—50 xylose units.

(2) *Colorimetric method.* Meyer's method (*Helv. Chim. Acta*, 1948, **31**, 103) was used. Standard curves were constructed for maltose (Meyer, *loc. cit.*) and for glucose and xylose, by treating known amounts of the sugars (0·1—2 mg.) with 3 : 5-dinitrosalicylic acid (1 c.c.; 1·5%) and sodium hydroxide (1 c.c.; 6N.) at 65° for 30 minutes, cooling, and diluting to 25 c.c. The solution thus obtained was compared with a blank in a "Spekker" photoelectric absorptiometer using the 1-cm. cell and filter 604 (see Table VI).

TABLE VI.

Sugar.	mg./ 25 c.c.	G.-mol./ 25 c.c. $\times 10^6$.	Log I/I_0.	Sugar.	mg./ 25 c.c.	G.-mol./ 25 c.c. $\times 10^6$.	Log I/I_0.	Sugar.	mg./ 25 c.c.	G.-mol./ 25 c.c. $\times 10^6$.	Log I/I_0.
Xylose	0·2	1·33	0·007	Glucose	0·2	1·11	0·005	Maltose	0·2	0·58	0·002
	0·3	2·0	0·016		0·3	1·66	0·015		0·5	1·46	0·020
	0·4	2·66	0·026		0·4	2·22	0·028		0·75	2·19	0·050
	0·5	3·33	0·053		0·5	2·77	0·046		1·0	2·92	0·091
	0·6	4·0	0·073		0·6	3·33	0·065		1·2	3·5	0·131
	0·7	4·66	0·102		0·7	3·88	0·094		1·5	4·38	0·197
	0·8	5·33	0·128		0·8	4·44	0·125		1·8	5·26	0·244
	0·9	5·99	0·164		0·9	4·99	0·158		2·0	5·84	0·298
	1·0	6·66	0·205		1·0	5·55	0·195				
	1·5	9·99	0·430		1·5	8·33	0·372				
	2·0	13·33	0·650		2·0	11·11	0·571				

The graphs constructed from these results give nearly parallel lines beyond a concentration of *ca.* $0·2 \times 10^{-6}$ g.-mol/25 c.c., with the glucose curve lying equidistant from the xylose and maltose curves. By analogy it is assumed that the hypothetical disaccharide related to xylan, as is maltose to starch, would give an absorption curve as far removed from that of xylose as is maltose from glucose, and this in fact would correspond with the experimental curve for glucose. For estimating the proportion of reducing groups in xylan, two equal samples (50—75 mg.) of xylan were dissolved in sodium hydroxide (1 c.c.; 6N.), and the same volume of 3 : 5-dinitrosalicylic acid was added to one of them, the nitrosalicylic acid for the blank being measured into a 25-c.c. standard flask. The volumes of both samples were made up to 7 c.c. with water and both samples were heated at 65° for 30 minutes. The solutions were cooled and made up to 25 c.c., and blank and unknown compared as before: 73·35 mg. of xylan gave log $I/I_0 = 0·30$, corresponding 60 $C_5H_8O_4$ units (xylose curve) or 72 units (glucose curve); 58·18 mg. of xylan gave log $I/I_0 = 0·172$, *i.e.*, 63 (xylose curve) or 79 units (glucose curve).

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THE UNIVERSITY, MANCHESTER.
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