153. *The Polysaccharide associated with β-Amylase.*

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The water-soluble polysaccharide associated with β-amylase in ungerminated wheat flour has been isolated and methylated. The polysaccharide is constituted of units of *l*-arabinose, *d*-xylose, and *d*-galactose. It contains no acidic residues.

Hydrolysis of the methylated polysaccharide yields trimethyl *l*-arabofuranose (6 mols.), 2 : 3-dimethyl xylose (6 mols.), 2-methyl xylose (1 mol.), xylose (1 mol.), and 2 : 4-dimethyl galactose (1 mol.).

The probable structure of the polysaccharide is discussed.

The free polysaccharide has no amylolytic activity.

In an investigation which will be published later on the constitution of a pure amylolytic enzyme, evidence accumulated that a polysaccharide was always associated with protein in the aqueous-alcoholic extracts of ungerminated cereals. These extracts have amylolytic activity of the β-type (Kuhn's nomenclature, *Annalen*, 1925, **433**, 1) and it became desirable to determine the nature of this cold-water-soluble polysaccharide and its functional relationship, if any, to the enzyme system.

Wroblewski (*Ber.*, 1897, **30**, 2289; 1898, **31**, 1130) demonstrated the presence in an aqueous-alcoholic extract of malt of a polysaccharide which was described as an araban for the reason that *l*-arabinosazone was prepared from it. The polysaccharide had, *per se*, no amylolytic activity. Most workers in this field, notably Sherman and his co-workers (cf. *J. Biol. Chem.*, 1934, **104**, 501), incline to the view that amylase is purely protein in character, although Pribram (*Biochem. Z.*, 1912, **44**, 293) regarded diastase as a polysaccharide acid in combination with a simple polypeptide and Itoh (*J. Biochem. Japan*, 1936, **23**, 125) concluded from spectroscopic observations that pancreatic amylase is carbohydrate rather than protein in nature. A similar view has been expressed by Akabori and Kasimoto (*Bull. Chem. Soc. Japan*, 1938, **13**, 291) in regard to taka-diastase.

We have obtained from the flour of ungerminated wheat, by extraction with cold aqueous alcohol, a β-amylase preparation which is free from α-amylase. The optimum concentration of alcohol for a large-scale preparation of the crude β-amylase is 20%. The extract contains carbohydrate and protein and its amylolytic activity is destroyed by heat, by dialysis, and by prolonged contact with alcohol. Evidence was obtained that the purest enzyme preparation probably contained no carbohydrate. The carbohydrate consists of dialysable free sugars and non-dialysable polysaccharide. It is with the latter that this paper is concerned.

Crude β-amylase, extracted as described, was freed from reducing sugars most advantageously by a process of controlled precipitation from aqueous solution with alcohol. The non-reducing product had $[\alpha]_D^{17°}$ −56° in water and gave tests for carbohydrate and protein. The polysaccharide was freed from protein by two procedures, by acetylation and by methylation. In the first case, it was possible to separate the polysaccharide acetate completely from the acetylated protein by extraction of the former with acetone. The nitrogen-free polysaccharide acetate showed $[\alpha]_D^{14°}$ − 57·7° in chloroform and an acetyl content corresponding to a diacetyl pentosan. Its de-acetylation by means of potassium hydroxide yielded the free polysaccharide. The latter was a white powder, easily soluble in cold water, in which it showed $[\alpha]_D^{20°}$ − 78·5°. It gave no colour with iodine and was not a starch or starch dextrin, it contained no acid groups and gave colour reactions indicating a pentosan structure. This protein-free polysaccharide showed no amylolytic activity.

The second method of purification, that of methylation, was not, of course, suitable for the preparation of the free polysaccharide. It was, however, utilised in the determination of the structure of the polysaccharide inasmuch as the treatment of the non-reducing amylase preparation with methyl sulphate and sodium hydroxide solution destroyed the protein constituent and gave the methylated polysaccharide in excellent yield.

The fully methylated polysaccharide was a pale yellow glass which gave negative tests for protein, nitrogen, and uronic acid. It was insoluble in water and showed $[\alpha]_D^{17°}$ − 107·5° in chloroform.

Rapid methanolysis of the methylated polysaccharide occurred when it was boiled with methyl-alcoholic hydrogen chloride and in the mixture of methyl glycosides so produced there were identified as principal constituents, trimethyl methyl-*l*-arabofuranoside and 2 : 3-dimethyl methyl-*d*-xylopyranoside.

The occurrence together of *l*-arabinose and *d*-xylose indicates either that the original polysaccharide is a mixture of an araban and a xylan or that it is homogeneous and contains units of *d*-xylose and *l*-arabinose as integral parts of its structure. If the first of these alternatives were true, it is to be expected that trimethyl xylose and dimethyl arabinose would form part of the hydrolysate. A careful search of the trimethyl pentose fraction based on the relative rates of hydrolysis of trimethyl methylarabinofuranoside and trimethyl methylxylopyranoside failed to reveal any of the latter compound. Similarly, the dimethyl pentose fraction appeared to consist exclusively of 2 : 3-dimethyl xylose.

When the methylated polysaccharide was prepared in larger quantity, it was possible to separate it into fractions by precipitation from chloroform solution with light petroleum. The fractions showed considerable variations in rotation ($[\alpha]_D$ − 75° to − 128°) and in viscosity in chloroform solution. Nevertheless the fractions showed the same rapid rate of methanolysis, and hydrolysis with aqueous acid of the methyl glycosides so produced yielded a mixture of methylated sugars which had the same reducing power in each case. Furthermore, distillation of the mixture of glycosides, prepared by the action of acid methyl alcohol, gave fractions with almost identical physical constants, irrespective of the methylated polysaccharide fraction from which they were obtained. It would seem, therefore, that the variations in rotation and viscosity referred to are due to differences of degree of aggregation of a repeating unit rather than to the chemical heterogeneity of the polysaccharide.

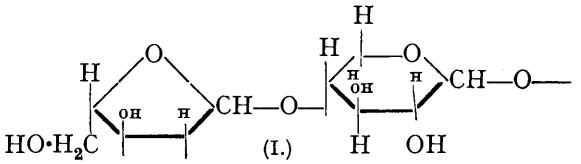
The mixture of methyl glycosides produced by the action of acid methyl alcohol on

each of the methylated polysaccharide fractions was fractionally distilled in a high vacuum. The first distillation fractions consisted essentially of trimethyl methyl-*l*-arabofuranoside, the middle fractions of 2 : 3-dimethyl methyl-*d*-xyloside. In the higher-boiling fractions were identified 2-methyl methylxyloside, methylxyloside and 2 : 4-dimethyl methyl-galactoside. It was possible to make an approximate evaluation of the relative molecular proportions of these constituents and it is concluded that the basal or repeating unit of the methylated polysaccharide is constituted of six trimethyl *l*-arabofuranose residues, six 2 : 3-dimethyl *d*-xylose residues, one *d*-xylose residue, one 2-methyl *d*-xylose residue, and one 2 : 4-dimethyl *d*-galactose residue.

One of these constituents, namely, the 2 : 4-dimethyl galactose, can only exist in the polysaccharide in the pyranose form, and the terminal arabinose units in the furanose form. There is not evidence at present available to indicate whether the xylose constituents exist in the pyranose or furanose forms, nor is it possible to decide the order and sequence of the linkage of the sugars constituting the repeating unit.

Certain general structural features may, however, be made out. The architectural plan on which the polysaccharide is built is much more complex than that of the linear polymers such as starch, cellulose or the xylan of esparto grass, and this complexity is expressed not only in the number of sugars (arabinose, xylose, and galactose) which go to form the repeating unit, but also in the different types which are displayed of mutual linkage of these sugars. In this respect, the polysaccharide bears some resemblance to the vegetable gums, although, unlike the latter, it contains no acid constituents.

It is clear that the *l*-arabinose constitutes terminal units exclusively and, inasmuch as the molecular proportions of trimethyl arabinose and dimethyl xylose are equal, it may be that six side chains of the disaccharide (I) radiate from a central nucleus or chain composed of three xylose units and one galactose unit. One of many ways in which this could occur is shown diagrammatically in (II).

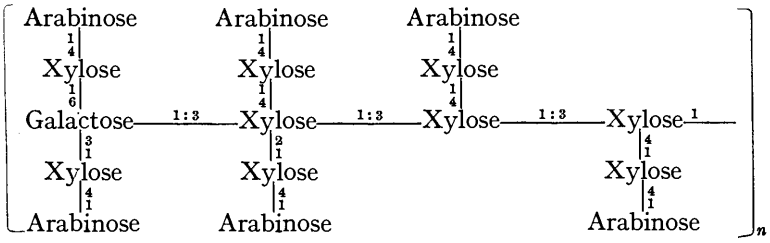


(I.)

Arabinose—(1:4)—Xylose—

(I*a*.)

(I*a*) is a diagrammatic representation of (I), the numerals indicating the positions of the mutual linking of sugar units. The same device is used also in (II).



(II.)

Experimental.

The Unit of Amylolytic Activity.—A known weight of the amylase preparation was allowed to act for 30 minutes at 35° on a soluble starch solution (0·6%) buffered to p_H 4·8. The enzyme was then destroyed by alkali and the reducing power of the resulting solution was estimated volumetrically by the method of Shaffer and Hartmann (*J. Biol. Chem.*, 1921, **45**, 365). The amylolytic activity of the enzyme is expressed as the number of mg. of maltose liberated from starch, under the standard conditions described, by 1 mg. of enzyme preparation.

Extraction of β-Amylase.—In a preliminary experiment to determine the optimal conditions of extraction, four batches (each 50 g.) of finely ground, ungerminated wheat were extracted by shaking for 12 hours at room temperature with (*a*) water, (*b*) 20% alcohol, (*c*) 50% alcohol, and (*d*) 70% alcohol. In each case the wheat residue was removed in a centrifuge and the

enzyme was precipitated by raising the concentration of alcohol to 80%. The precipitates were separated and dried in a vacuum and the amylolytic activity of each was determined with the following results :

Aqueous-alcohol extractant. Conc. of alcohol (%).	Weight of amylase (g.).	Amylolytic activity (units).
0	0·7	22
20	0·6	30
50	0·5	19·5
70	0·45	2

It is obvious that the most active preparation is that obtained with 20% alcohol–water and this extractant was used for the preparation of β-amylase in quantity. By the extraction of 1000 g. of wheat flour with 2·5 l. of 20% alcohol there were obtained 13·5 g. of a preparation with an activity of 30 units. The crude β-amylase so prepared was free from α-amylase (as shown by the Wijsmann test). It contained reducing sugars and gave strong colour reactions for protein. It gave no colour with iodine, indicating the absence of starch and the higher starch dextrins. It contained nitrogen, phosphorus and sulphur but no halogen. Part of the phosphorus was present as inorganic phosphate (Bell–Doisy test). The preparation was not completely soluble in water. The insoluble residue gave only faint carbohydrate reactions and would appear to consist of protein denatured during the precipitation with alcohol.

The crude β-amylase was freed from reducing sugars in the following way. The amylase (40 g.) was shaken with water (800 c.c.), and the insoluble portion removed. Alcohol (4 l.) was added to the filtrate with vigorous stirring. The precipitate was separated in a centrifuge, washed with alcohol and ether, and dried in a vacuum. Yield, 24 g. This product was non-reducing to Fehling's solution, with which it formed a soluble violet complex. It gave protein reactions but no colour with iodine. The naphtharesorcinol test for uronic acid was negative, although on boiling the amylase with hydrochloric acid furfural was produced and aniline acetate paper was stained red, an indication of the presence of pentosans. This non-reducing β-amylase preparation, which showed $[\alpha]_D^{17°}$ — 56·1° in water (*c*, 0·7), was used in the subsequent experiments.

Acetylation of β-Amylase.—The non-reducing amylase (5 g.), prepared as described, was dissolved in the minimum amount of water (7 c.c.) and to this solution were added pyridine (100 c.c.) and acetic anhydride (150 c.c.). The solution was refluxed at 80° for 2 hours, cooled, and decanted from a small amount of undissolved material into ice-water (2 l.). After being kept overnight, the precipitate was separated in a centrifuge and was washed with water until it was no longer acid to Congo-red. Thereafter the product was washed with cold alcohol and ether and was dried in a vacuum at 60°. Yield, 3·2 g. The alcoholic washings gave a solid (1·8 g.) on evaporation. This material contained nitrogen and only a small proportion of carbohydrate; it was not further examined. The alcohol-insoluble product also contained nitrogen. It was extracted with warm acetone until the last extract gave only a faint Molisch reaction. The combined extract was concentrated to about 50 c.c., and light petroleum added until a small permanent precipitate was formed. This was removed in a centrifuge and the clear mother-liquor was poured slowly and with stirring into light petroleum (400 c.c.). The precipitate was collected on a filter and dried in a vacuum at 50°. It was non-reducing, contained no nitrogen, and showed $[\alpha]_D^{14°}$ — 57·7° in chloroform (*c*, 0·9) (Found : CH_3·CO, 40·2. Diacetyl pentosan requires CH_3·CO, 39·8%).

Deacetylation. The acetate (1·2 g.) was dissolved in acetone, and 10% aqueous potassium hydroxide (12 c.c.) added. Two layers persisted and after 2 hours the upper (acetone) layer was removed, and the aqueous layer, diluted with water, was stirred into alcohol. The precipitate was separated and dissolved in water; the solution, made faintly acid with acetic acid, was precipitated by the addition of alcohol. The product was reprecipitated in the same way and was finally washed with alcohol and ether and dried in a vacuum at 50°. Yield 0·54 g. The polysaccharide was non-reducing, contained no nitrogen, and showed $[\alpha]_D^{20°}$ — 78·5° in water (*c*, 1·0); its equivalent by titration was 9325, indicating a negligible proportion of acid groups. It showed no amylolytic activity.

The Methylation of β-Amylase.—Three batches of methylated polysaccharide were separately prepared and examined, but, as the examinations were mainly repetitive, it will be necessary to record in full the investigation of only two of these batches, namely, II and III. Examination of batch I (2·5 g.) revealed that the methylated polysaccharide was easily hydrolysable by the agency of acid methyl alcohol and among the products of hydrolysis were trimethyl arabinose, dimethyl xylose, and probably a dimethyl hexose.

Methylated polysaccharide, batch II. Preparation. Non-reducing β-amylase (20 g.) was suspended in water and at 40°, methyl sulphate (200 c.c.) and 30% sodium hydroxide solution were added in $\frac{1}{10}$ portions at 20-minute intervals. After being stirred for several hours, the mixture was heated on a boiling water-bath for ½ hour and, after being cooled, was brought to a faint alkalinity with sulphuric acid. It was then raised to the b. p. and filtered while hot through cloth. Complete methylation was achieved after five treatments, in the last two of which acetone was added as a solvent. As the methylated polysaccharide was somewhat soluble in cold water, it could not be purified by washing. It was freed from inorganic matter by being shaken together with a large quantity of anhydrous magnesium sulphate in chloroform. After several hours the methylated compound had dissolved and the solution was filtered and evaporated. The product was a pale yellow glass soluble in chloroform, moist acetone and methyl iodide but insoluble in hot water, methyl alcohol, ether or petroleum. It was free from nitrogen and protein. This product was methylated further by treatment with methyl iodide and dry silver oxide; the methylated product so obtained showed OMe, 39·0 and ash, 0·3% (Calc. for dimethyl pentosan: OMe, 39·8%). Yield, 13·3 g.

The methylated polysaccharide so prepared (25 g.) was separated into fractions by the graduated addition of light petroleum to its solution in chloroform. The fractions had the following properties:

Fraction.	Weight, g.	OMe, %.	Ash, %.	$[\alpha]_D^{14°}$ in $CHCl_3$.
A	0·25	39·0	0·6	—
B	1·5	38·4	0·4	—
C	19·9	39·3	0·02	−85·9°

Methanolysis. Fractions B and C were combined (21·5 g.) and refluxed with 4% methyl-alcoholic hydrogen chloride (400 c.c.) for 10 hours. The solution was then neutralised with silver carbonate and filtered, and the methyl alcohol distilled from the filtrate at atmospheric pressure (a trimethyl methylpentoside is volatile in methyl alcohol at low pressures). The non-reducing syrup so obtained was submitted to fractional distillation from a Widmer flask at 20 mm. pressure for the first fractions and subsequently in a high vacuum:

TABLE I.

Fraction.	Bath temp.	Pressure, mm.	Weight, g.	OMe, %.	$[\alpha]_D$ in water.	$n_D^{22·5°}$
1	135°	20	0·08	50·0	− 35·4°	1·4310
2	135	20	0·20	54·8	− 72·3	1·4322
3	135—140	20	5·36	59·4	− 78·3	1·4328
4	140—145	20	0·94	58·6	− 53·0	1·4338
5	103—110	0·04	1·05	59·7	+ 4·2	1·4393
6	108—120	0·04	6·18	50·9	+ 32·2	1·4510
7	123—135	0·04	1·80	47·9	+ 82·6	1·4565
8	135—147	0·04	1·61	44·9	+ 68·3	1·4620
9	142—147	0·01	1·52	39·5	+100·0	1·4750
10	155—200	0·01	2·05	33·6	+105·0	1·4817
11	Residue	—	0·89	—	—	—

Inasmuch as a tri-, di-, and mono-methyl methylpentoside have 60·2, 48·4, and 34·8% OMe respectively, it would appear from the methoxyl contents that fractions 3, 4, and 5 consist entirely of trimethyl methylpentoside and that the chief constituent of fractions 6 and 7 is a dimethyl methylpentoside. The low refractive indices and methoxyl contents of fractions 1 and 2 suggest the presence therein of methyl lævulate and this in fact was confirmed by the treatment of the combined fractions 1 and 2 (0·20 g.) with N/10-barium hydroxide (30 c.c.) at 80° for 2 hours. After neutralisation with carbon dioxide and filtration, the filtrate was exhaustively extracted with chloroform. The extract (0·135 g.), distilled (water-pump), yielded a syrup which had constants almost identical with those of fraction 3, namely, $[\alpha]_D^{12°}$ − 82·2° in water, $n_D^{20°}$ 1·4350, and OMe 58·2%.

Examination of the trimethyl methylpentoside fractions 3, 4, *and* 5. The possible existence of trimethyl xylose as an end group in addition to trimethyl arabofuranose has been discussed in the introduction. The trimethyl methylpentoside fractions were therefore examined with this possibility in mind.

Fraction 3 (1·01 g.) was dissolved in N/25-hydrobromic acid (50 c.c.) and heated on the boiling water-bath. It is to be expected that only methylfuranosides will be hydrolysed by acid of this strength and that any methylpyranosides present will remain unchanged. The hydrolysis was followed polarimetrically: [α], calc. on initial weight, − 82·6° (initial value),

— 77·5° (30 mins.), — 56·2° (205 mins.), — 44·8° (380 mins.), — 36·8° (565 mins.), — 34·7° (685 mins.), — 33·7° (705 mins). The solution was neutralised with silver carbonate, and the product (0·83 g.) extracted with ether. It showed $[\alpha]_D^{16°}$ — 35·4° in water (*c*, 0·8).

The syrupy product was now heated on a boiling water-bath with N-sulphuric acid for 5 hours. No change in rotation occurred during this time, from which it is concluded that no methylpyranoside was present. Isolation of the product from the acid solution gave a syrup (0·64 g.) which had $[\alpha]_D^{18°}$ — 34·4° in water (*c*, 1·1) and OMe 47·3% (Calc. for trimethyl pentose : OMe, 48·4%).

The syrup, dissolved in water, was kept in contact with bromine (1·5 c.c.) for 1 hour at 50° and for 3 days at room temperature. The solution was then non-reducing and excess of bromine was removed by aeration. The solution was neutralised with silver carbonate and, after filtration, the silver salt of trimethyl pentonic acid was decomposed with hydrogen sulphide. The solution was evaporated to dryness, and the lactone extracted with ether. The syrup (0·42 g.) so obtained, after distillation at bath temperature 110—140°/0·03 mm., crystallised completely when nucleated with 2 : 3 : 5-trimethyl *l*-arabonolactone. This was identical with a similar preparation from fraction 4, which was a low-melting solid showing $[\alpha]_D^{13°}$ — 19·1° in methyl alcohol (*c*, 1·8) and $n_D^{16°}$ 1·4440.

The constitution of the lactone was confirmed by conversion into the corresponding amide. The lactone was dissolved in dry methyl alcohol, and the solution saturated with ammonia at 0°. After 2 days, the solution was evaporated, and the amide crystallised. It was recrystallised from ethyl acetate–light petroleum and then showed m. p. 136—137°. In admixture with authentic 2 : 3 : 5-trimethyl *l*-arabonamide no depression of m. p. occurred. The amide had $[\alpha]_D^{18°}$ 22·7° in ethyl alcohol (*c*, 2·0) (Found : C, 46·4; H, 7·6; N, 6·7; OMe, 44·1. Calc. for $C_8H_{17}O_5N$: C, 46·4; H, 8·2; N, 6·8; OMe, 44·9%).

It is clear that fraction 3 consists entirely of trimethyl methylarabofuranoside. By a similar procedure it was shown that fractions 4 and 5 were free from trimethyl methylxyloside and consisted solely of the fully methylated arabofuranose.

Examination of fraction 6 (*Table I*). The refractive index and methoxyl content of fraction 6 suggest that it is a mixture of tri- and di-methyl methylpentosides. It was fractionally distilled at 0·03 mm. from a Widmer flask :

Fraction.	Bath temp.	Weight, g.	OMe, %.	$[\alpha]_D$ in water.	Fraction.	Bath temp.	Weight, g.	OMe, %.	$[\alpha]_D$ in water.
6*a*	108—110°	1·12	51·0	+27·7°	6*d*	110—150°	0·35	48·8	+73·4°
6*b*	110—120	2·87	49·1	+43·0	6*e*	150—220	0·14	44·5	+38·0
6*c*	120—150	0·82	48·5	+73·8	6*f*	Residue	0·39	—	—

Fractions 6*c* and 6*d* are identical and have the constants of dimethyl methylpentoside (OMe, 48·4%). They were combined (1·15 g.) and heated on a boiling water-bath with N/15-hydrobromic acid (100 c.c.) : $[\alpha]_D$ + 83·5° (initial value), + 82·0° (70 mins.), + 78·3° (180 mins.), + 71·5° (425 mins.), + 66·2° (530 mins.), + 57·5° (770 mins.), + 50·5° (1140 mins.), + 47·0° (1240 mins.).

The figures show that hydrolysis proceeds very slowly with this strength of acid and is not complete after 21 hours, an indication of the pyranoside structure of the dimethyl methylpentoside. The acid strength was now increased to N, and the heating continued. The final constant value of $[\alpha]_D$ + 33·1° was attained after 3½ hours. The product, worked up in the usual way, was a syrup showing $n_D^{15·5°}$ 1·4668 and $[\alpha]_D^{16°}$ + 30·9° in water (*c*, 0·9) (Found : OMe, 35·4. Calc. for dimethyl pentose : OMe, 34·8%).

Oxidation of the dimethyl pentose (0·75 g.) with bromine water yielded a syrup (0·44 g.), which, after distillation, had OMe, 37·0% (Calc. for dimethyl pentonolactone : OMe, 35·4%). The following changes were observed when the lactone was dissolved in water : $[\alpha]_D$ + 95·9° (initial value), + 90·5° (13 hrs.), + 88·7° (28 hrs.), + 86·9° (35 hrs.), + 77·9° (123 hrs.), + 70·6° (266 hrs.), + 67·1° (388 hrs.).

These figures are almost identical with those recorded by Hampton, Haworth, and Hirst (*loc. cit.*) for the hydration of 2 : 3-dimethyl γ-xylonolactone in water.

The constitution was confirmed by the conversion of the lactone into the corresponding crystalline amide, $[\alpha]_D^{16°}$ + 49·1° in water (*c*, 0·4), m. p. 134°, not depressed by authentic 2 : 3-dimethyl xylonamide.

The dimethyl pentose (0·10 g.) was converted into the anilide by boiling with an alcoholic solution of aniline. The anilide showed m. p. 129° and $[\alpha]_D^{18°}$ + 194° in ethyl acetate (*c*, 0·5). Authentic 2 : 3-dimethyl xylose anilide was prepared and showed m. p. 133° and $[\alpha]_D^{18°}$ + 197°

in ethyl acetate (*c*, 1·2). When mixed, these anilides had m. p. 130—131° (Hampton, Haworth, and Hirst record 146° as the m. p. of the anilide). It is concluded that fractions 6*c* and 6*d* consist essentially of 2 : 3-dimethyl methylxyloside.

Examination of fractions 8, 9, 10 *and* 11 (*Table I*). The higher fractions might conceivably contain partially hydrolysed material. Fractions 8, 9, 10 and the undistilled residue were therefore combined (5·72 g.) and submitted to the further action of boiling 4% methyl-alcoholic hydrogen chloride for 14 hours. The product (5·55 g.), isolated as previously described, was fractionally distilled at 0·01 mm. :

Fraction.	Bath temp.	Weight, g.	OMe, %.	$[\alpha]_D$ in water.	Fraction.	Bath temp.	Weight, g.	OMe, %.	$[\alpha]_D$ in water.
8*a*	125—128°	0·41	38·3	—	8*e*	148—160°	1·07	34·4	+102°
8*b*	127—132	1·40	33·8	+ 65·6°	8*f*	160—200	0·46	30·9	+ 85·3
8*c*	132—145	0·47	34·0	+ 89·0	8*g*	Residue	0·49	—	—
8*d*	145—148	1·02	32·9	+109					

The methoxyl contents of fractions 8*b*—8*e* correspond to that of a monomethyl methylpentoside (34·8%).

Fraction 8*c* (0·42 g.) was heated on a boiling water-bath with N/25-hydrobromic acid. As no hydrolysis occurred (indicating the absence of a furanoside), the acid strength was increased to N by the addition of sulphuric acid. Hydrolysis was complete in 5 hours ($[\alpha]_D$ + 98·0° ⟶ + 69·2°) and the product isolated was a syrup (0·318 g.) containing OMe, 27·9% (calc. for monomethyl pentose: OMe, 18·9; for dimethyl hexose, OMe, 29·8%). The sugar partly crystallised after some days and the crystals were separated from syrup (S) by treatment with light petroleum–ether–alcohol. Recrystallised from ethyl acetate, the crystalline portion melted at 129—131°, not depressed by authentic 2-methyl xylose.

The syrup S (60 mg.) was converted into the corrresponding glycoside by being boiled with 2% methyl-alcoholic hydrogen chloride. The glycoside was then methylated by treatment with methyl iodide and silver oxide. The product had OMe, 61·0% and therefore probably contained tetramethyl methylhexoside (OMe, 62·0%) in addition to trimethyl methylxyloside (OMe, 60·2%).

Fraction 8*d* had an equivalent of 6000, indicating the absence of a significant amount of acid residues. After several months, partial crystallisation occurred. The crystalline portion, separated by treatment with ether–alcohol, was recrystallised from acetone–ether–light petroleum and then showed $[\alpha]_D^{20°}$ + 2·0° in water (*c*, 1·0) (Found: OMe, 40·6. Calc. for dimethyl methylhexoside: OMe, 41·9%), m. p. 168°, not depressed by authentic 2 : 4-dimethyl β-methylgalactoside.

On the basis of the equilibrium value of $[\alpha]_D$ for fraction 8*d* after hydrolysis (+ 54·5°) it was calculated that this hydrolysed fraction contained 58% of 2 : 4-dimethyl galactose ($[\alpha]_D$ 86°) and 42% of 2-methyl xylose.

Methylated polysaccharide, batch III. β-Amylase (activity 30 units) was methylated by essentially the same procedure as that used for batch II. From 180 g. of β-amylase there were obtained 108 g. of methylated polysaccharide (OMe, 38·5%). The final treatment with methyl iodide and silver oxide was omitted because the absence of acid residues in the polysaccharide had been clearly demonstrated.

Batch III was separated into fractions by the graduated addition of light petroleum to its solution in chloroform. The fractions had the following properties :

Fraction.	Weight, g.	Ash, %.	OMe, %, corr. for ash.	$[\alpha]_D$ in $CHCl_3$.	$\eta_{sp.}/c$ in $CHCl_3$.
A	5·9	7·20	39·1	− 86°	—
B	18·6	2·02	39·0	−103	0·398
C	23·9	0·13	38·1	−128	0·840
D	14·3	0·00	38·6	−111	0·369
E	17·6	0·18	40·0	− 75	0·164

Uronic acid content. Estimation of the carbon dioxide formed when the methylated polysaccharide was boiled with 12% hydrochloric acid confirmed the absence of uronic acid residues. Fractions B, C, D and E gave respectively 0·37, 0·52, 0·11 and 0·56% of carbon dioxide (cf. Campbell, Hirst, and Young, *Nature*, 1938, **142**, 912).

Comparative hydrolysis of fractions C and D. Despite the great difference in specific viscosity shown by C and D there is reason to believe that chemically those two fractions are identical. The mixture of sugars obtained when C was hydrolysed initially with 4% methyl-alcoholic

hydrogen chloride and subsequently with sulphuric acid showed $[\alpha]_D$ + 6·1° (equilibrium value) and copper number (by the Shaffer–Hartmann method), 24·3. Fraction D, similarly treated, yielded a mixture of sugars having $[\alpha]_D$ + 12·3° and copper number, 24·5.

Methanolysis of the methylated polysaccharide (fractions C and D). Fractions C (24·21 g.) and D (12·66 g.) were separately boiled with 4% methyl-alcoholic hydrogen chloride and the products fractionally distilled :

TABLE II.

Glycosides from fraction C.

Fraction.	Bath temp.	Pressure, mm.	Weight, g.	$n_D^{20°}$.
C1	155°	20	0·29	1·4340
C2	156—165	20	8·04	1·4350
C3	94—96	0·02	1·465	1·4375
C4	97—101	0·01	0·30	1·4495
C5	103—124	0·02	6·17	1·4545
C6	125—134	0·02	0·87	1·4560
C7	135—150	0·02	1·09	1·4650

TABLE III.

Glycosides from fraction D.

Fraction.	Bath temp.	Pressure, mm.	Weight, g.	$n_{20°}^{D}$.
D1	148°	20	0·09	1·4340
D2	150—160	20	3·54	1·4350
D3	160—165	20	1·20	1·4395
D4	95—98	0·02	0·59	1·4485
D5	105—125	0·02	3·29	1·4535
D6	130—150	0·02	0·85	1·4590

The fractions from C and D are nearly identical. For that reason the undistilled residues in the above fractionations (Tables II and III) were combined and the distillation continued.

TABLE IV.

Glycosides from fractions C and D.

Fraction.	Bath temp.	Pressure, mm.	Weight, g.	$n_D^{20°}$.	OMe, %.	$[\alpha]_D^{17°}$ in water.
CD8	155—160°	0·02	0·93	1·4706	37·8	—
CD9	160—161	0·02	1·40	1·4772	36·1	+103°
CD10	161—175	0·02	2·11	1·4830	31·3	—

The undistilled residue was submitted to the further action of boiling 4% methyl-alcoholic hydrogen chloride (150 c.c.) for 8 hours. The product was distilled at 0·02 mm. :

Fraction.	Bath temp.	Weight, g.	$n_D^{20°}$.	OMe, %.
CD11	100—130°	0·94	1·4420 to 1·4720	—
CD12	135—180	1·39	1·4850	33·7
CD13	Residue	0·74	—	—

Examination of fraction CD9 (Table IV). Hydrolysis of fraction CD9 (0·81 g.) with N-sulphuric acid yielded a syrup (0·58 g.) which showed $[\alpha]_D^{17°}$ + 55·4°, equilibrium value in water (*c*, 1·1). If the fraction was a mixture of 2 : 4-dimethyl galactose ($[\alpha]_D$ + 86°) and 2-methyl xylose ($[\alpha]_D$ + 34°), it could be calculated that the dimethyl galactose represented 40% of the mixture.

Methylation of fraction CD9 (0·475 g.) with methyl iodide and silver oxide yielded a syrup, which was fractionally distilled, giving fraction 1 (0·40 g.), b. p. (bath temp.) 125—135°/20 mm., $n_D^{18°}$, 1·4423, $[\alpha]_D^{16°}$ in water + 86·3°, and fraction 2 (0·02 g.), b. p. 110—170°/0·03 mm., $n_D^{18°}$ 1·4485. From the refractive index of fraction 1 it is calculated to contain (roughly) 36% of tetramethyl methylgalactoside and 64% of trimethyl methylxyloside. After hydrolysis, fraction 1 yielded crystalline trimethyl xylopyranose (m. p. 88—89°) and crystalline tetramethyl galactopyranose anilide (m. p. 190°). Fraction 2 was composed entirely of tetramethyl methylgalactoside, for it was possible to convert it quantitatively into tetramethyl galactose anilide.

Examination of fraction CD10. Hydrolysis of fraction CD10 (0·735 g.) was carried out with hot N-sulphuric acid (500 c.c.). The solution was then neutralised with barium carbonate, filtered, and evaporated to dryness. The residue was extracted first with boiling chloroform and then with boiling ethyl alcohol. The chloroform extract gave a syrup (0·235 g.) which showed $[\alpha]_D^{13°}$ + 61·2° in water (*c*, 0·7). The alcoholic extract also yielded a syrup (0·42 g.), which partly crystallised on trituration with cold alcohol. The crystalline portion was α-*d*-xylose, which, after recrystallisation from ethyl acetate, had m. p. and mixed m. p. 138—140° and $[\alpha]_D$ + 64·3° (5 mins.) ⟶ + 19·8° (equilibrium value in water). The syrup (0·33 g.) remaining after the separation of the crystals had $[\alpha]_D^{20°}$ + 35·3° in water (*c*, 0·7).

The methylation of fraction CD10 with methyl iodide and silver oxide yielded a syrup, which was distilled, giving fraction *a* (0·375 g.), $n_D^{17°}$ 1·4425, $[\alpha]_D^{18°}$ in water + 87·0°, and fraction *b* (0·084 g.), $n_D^{17°}$ 1·4500.

Hydrolysis of fraction *a* with N-sulphuric acid gave a syrup (0·28 g.) which showed $[\alpha]_D^{16°}$ + 55·3° in water (*c*, 1·4) and from which were obtained trimethyl xylose (m. p. 89°) and tetramethyl galactose anilide. Fraction *b* consisted entirely of tetramethyl methylgalactoside.

It was possible to make an approximate estimation of the relative proportions of the constituents of the glycoside mixture from the methylated polysaccharide.

The relative proportions of trimethyl arabinose and dimethyl xylose were calculated from the data in Table I, as follows :

Fraction.	1 and 2.	3.	4.	5.	6.	7.	8.	9.	10.
Trimethyl methylarabinoside, g.	0·2	5·4	0·9	1·0	1·5	—	—	—	—
Dimethyl methylxyloside, g.	—	—	—	—	4·7	1·8	1·3	0·5	0·1

The total weights are thus 9·0 g. of trimethyl methylarabinoside and 8·4 g. of dimethyl methylxyloside. These weights correspond to a molecular ratio of 1 : 1.

In the same way, from the data in Tables II, III, and IV the following calculations were made :

Fraction.	C4.	C5.	C6.	C7.	D4.	D5.	D6.	CD8.	CD9.	CD10.	CD12.
Dimethyl methylxyloside, g.	0·2	5·6	0·9	0·7	0·4	3·0	0·7	0·1	—	—	—
Monomethyl methylxyloside, g.	—	—	—	0·4	—	—	0·1	0·5	0·8	—	—
Dimethyl methylgalactoside, g.	—	—	—	—	—	—	—	0·3	0·6	1·0	0·9
Methyl xyloside, g.	—	—	—	—	—	—	—	—	—	1·1	0·5

The total weights are thus dimethyl methylxyloside 11·6 g., monomethyl methylxyloside 1·8 g., dimethyl methylgalactoside 2·8 g., methyl xyloside 1·6 g., corresponding approximately to the molecular proportions 6 : 1 : 1 : 1.

It is thus seen that the molecular proportions are (very approximately) trimethyl arabofuranose 6 mols., 2 : 3-dimethyl xylose 6 mols., 2-methyl xylose 1 mol., dimethyl galactose 1 mol., and xylose 1 mol.

The authors are indebted to Professor W. N. Haworth, F.R.S., for his interest.

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[*Received, October 24th*, 1941.]