615. *Studies of* Aspergillus niger. *Part I. The Structure of the Polyglucosan synthesised by* Aspergillus niger 152.

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"Mycodextran," renamed "nigeran," an intracellular polysaccharide of *Aspergillus niger* (strain 152), is a unique, essentially unbranched polyglucosan, in which about half of the glucosidic linkages are α-1 : 4, and half α-1 : 3; a small proportion of 1 : 6-bonds also may be present. End-group assay revealed an average chain length of 300—350 anhydroglucose units. Most, if not all, of the α-1 : 4- and α-1 : 3-linkages are arranged alternately. The name "nigerose" is suggested for 3-*O*-α-D-glucopyranosyl-D-glucose.

In 1914, Dox and Niedig (*J. Biol. Chem.*, 1914, **18**, 167) reported that a polyglucosan ("mycodextran"), having $[\alpha]_D^{20}$ +251° in 0·1N-sodium hydroxide, can be extracted from *Penicillium expansum* with hot water; it is deposited when the solution is cooled. A similar polysaccharide was isolated by Dox (*ibid.*, 1915, **20**, 83) from cultures of *Aspergillus niger*. The present paper describes studies, previously reported briefly (Barker, Bourne, and Stacey, *Chem. and Ind.*, 1952, 756), of a polyglucosan, which was probably identical with these aforementioned, and which had been synthesised from sucrose by *Aspergillus niger* (strain 152). Two samples (A and B) of the polysaccharide were kindly provided by Dr. J. L. Yuill; sample (A) was the material which had been deposited when four successive hot-water extracts of the washed mycelium had been combined and cooled, while sample (B) was a purer product derived from only the last two of four such extracts. Dr. Yuill had shown previously (*ibid.*, p. 755) that analyses of sample (B), which was free from nitrogenous and mineral matter, indicated a formula $(C_6H_{10}O_5)_n$.

Paper chromatographic analysis of a hydrolysate of sample (B) revealed a single aldose component; this had an R_F value identical with that of glucose. The extent of the conversion into glucose, determined by cuprimetric titration, was 97—98%. Sample (B), which gave a triacetate with acetic anhydride in pyridine, had $[\alpha]_D^{16}$ +283° in N-sodium hydroxide, compared with +270° for sample (A); these are exceptionally high figures for polyglucosans.

Samples (A) and (B) were partially hydrolysed with sulphuric acid, and the products

were analysed by paper chromatography; in each case the probable sugar components were those shown in the Table; the numerical results in the Table are those obtained when the hydrolysate from sample (B) was fractionated with aqueous ethanol on a charcoal column (cf. Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677; Bailey, Whelan, and Peat, *J.*, 1950, 3692).

Solvent used for elution	Wt. (g.) of product	$[\alpha]_D$ (equil.) in water	Probable sugar components (by paper chromatography)	Conversion (%) into glucose by acid
Water, 1200 c.c.	0·749	+53°	Glucose	100
5% Ethanol (1), 900 c.c. ...	0·003	—	*iso*Maltose, maltose	—
5% Ethanol (2), 1000 c.c....	0·256	+134	Maltose, disaccharide (X)	94
10% Ethanol, 550 c.c.	0·031	+136	Disaccharide (X)	—
15% Ethanol, 1000 c.c. ...	0·068	+160	Trisaccharide	94
35% Ethanol, 400 c.c.	0·216	+197	Higher saccharides	99

The monosaccharide fraction gave crystalline α-D-glucose in 49% yield, and from the residual syrup D-glucosazone, D-glucophenylosotriazole, and β-D-glucopyranose penta-acetate were obtained. The first 5% ethanol eluate contained two disaccharides, probably *iso*maltose and maltose; the former, which constituted <1% of the total disaccharides isolated, may have arisen from α-1 : 6-linked glucose units in the polysaccharide, or it may have been a reversion product. The two principal bioses produced were maltose (characterised as its β-octa-acetate), and a disaccharide (X) having an R_F value almost identical with that of laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucopyranose); its large equilibrium optical rotation supported the view that an α-glucosidic linkage was present. Three crystalline derivatives of (X) were prepared, namely, an octa-acetate, the phenylosazone, and the phenylosotriazole, the last two being identical with the corresponding derivatives of turanose. Thus (X) was 3-*O*-α-D-glucopyranosyl-D-glucose. [For proof that turanose is 3-*O*-α-D-glucopyranosyl-D-fructose see Hudson's review (*Adv. Carbohydrate Chem.*, 1946, **2**, 1).] Gakhokidze (*J. Gen. Chem., U.S.S.R.*, 1946, **16**, 1923; 1949, **19**, 571, 2100) claimed to have synthesised crystalline 3-*O*-α-D-glucopyranosyl-D-glucopyranose by condensing α-D-glucopyranose 2 : 3 : 4 : 6-tetra-acetate with 4 : 6-*O*-benzylidene-1 : 2-*O*-*iso*propylidene-D-glucopyranose, and then removing the protecting groups; he gave $[\alpha]_D^{18}$ +84·8° (in H_2O), but did not indicate whether this was the initial or equilibrium value (see Evans, Reynolds, and Talley, *Adv. Carbohydrate Chem.*, 1951, **6**, 27).

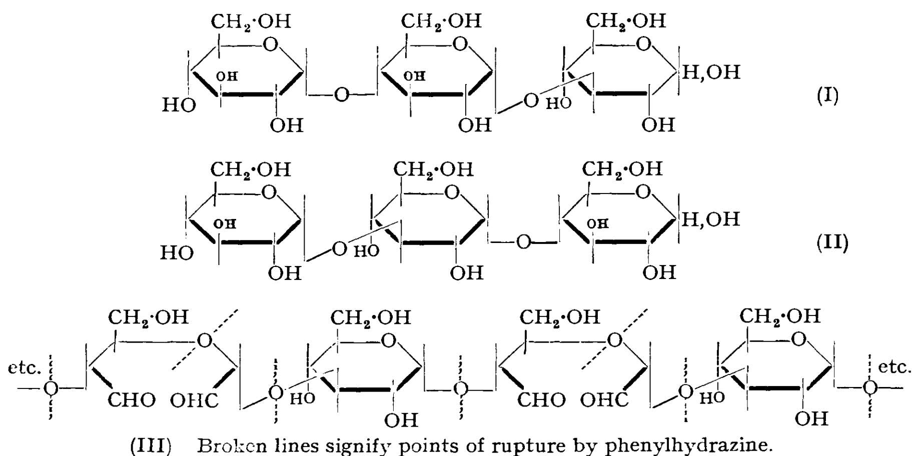
Methylations of samples (A) and (B) with sodium and methyl iodide in liquid ammonia afforded products with OMe, 44·6 and 45·0%, respectively (a tri-*O*-methylglucosan requires OMe, 45·6%). Filter-paper chromatography indicated that the two trimethyl ethers gave the same sugars when hydrolysed. In each case, there were two principal sugar components having R_F values and staining properties with aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443) identical with those of 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methyl-D-glucose. No di-*O*-methyl-D-glucose was detected, but a trace of a third component was shown, after being enriched by partition with chloroform, to be indistinguishable in its mobility from 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. By use of the same partition technique, followed by quantitative assay on filter paper (Hirst, Hough, and Jones, *J.*, 1949, 928), the average chain length of the methyl ether of sample (A) was determined as *ca.* 300—350 glucose units. An approximate value of 175 glucose units was obtained when the degree of polymerisation of sample (B) was determined with an alkaline solution of 3 : 5-dinitrosalicylic acid (cf. Meyer, Bernfeld, Boissonnas, Gürtler, and Noelting, *J. Phys. Colloid Chem.*, 1949, **53**, 319). This lower figure may have been due, in part, to the known alkali-lability of reducing glucose units linked through position 3 (cf. Corbett, Kenner, and Richards, *Chem. and Ind.*, 1953, 154).

Fractional crystallisation of a hydrolysate of methylated sample (A) from ether afforded 2 : 4 : 6-tri-*O*-methyl-α-D-glucose; a further quantity of this sugar was recovered as its crystalline aniline derivative. The mother-liquors from this preparation, after being hydrolysed with acid, yielded 2 : 3 : 6-tri-*O*-methyl-α-D-glucose, which was isolated also as its αβ-1 : 4-bisphenylazobenzoate (cf. Coleman, Rees, Sundberg, and McCloskey, *J. Amer. Chem. Soc.*, 1945, **67**, 381). Since 2 : 3 : 6-tri-*O*-methyl-D-glucose differs from all other trimethyl ethers of glucopyranose in its ability to form a methylfuranoside, the

ratio of the two principal sugar derivatives in the hydrolysate of methylated sample (A) could be determined from measurements of the optical rotation of the mixture in cold 2% methanolic hydrogen chloride (cf. Granichstädten and Percival, *J.*, 1943, 54); the observed fall in the value of $[\alpha]_D$ from +70·8° to +22·6° corresponded to that shown by a mixture containing 47% of the 2 : 3 : 6-tri-*O*-methyl sugar.

Thus it was evident that, in the polyglucosan under examination, *ca.* 47% of the glucose units were linked through positions 1 and 4, and *ca.* 53% through positions 1 and 3. Hence, only *ca.* 47% of the units should have been susceptible to periodate oxidation, as was in fact confirmed subsequently, 0·47 and 0·48 molecular proportion of the oxidant being consumed per anhydroglucose unit in sample (B) after 44 and 92 hours, respectively. The periodate-resistant units yielded glucose on hydrolysis. The very small amount (*ca.* 0·03 molecular proportion per anhydroglucose unit) of formic acid liberated during the oxidation was consistent with this view, but no attempt was made to assess the chain length of the polysaccharide on this basis, because of (*a*) the difficulties inherent in determinations of such small amounts of the acid in the presence of other oxidation products, (*b*) dubiety concerning the number of mols. of formic acid liberated from the ends of an unbranched polysaccharide chain, and (*c*) the possibility that a small proportion of 1 : 6-linkages may have occurred in the polysaccharide (see above).

The failure of " mycodextran " to give a stain with iodine, and the resistance of the higher saccharides mentioned in the Table to both α- and β-amylase, revealed that the polysaccharide was not a mixture of amylose and an α-1 : 3-linked glucosan; moreover there could not have been any significant proportion of uninterrupted sequences of α-1 : 4-linkages more than *ca.* ten glucose units in length. As has been described by Bayly and Bourne (*Nature*, 1953, **171**, 385), the trisaccharide fraction mentioned in the Table was examined by the sensitive paper-chromatographic technique, involving conversion on the paper into its benzylamine derivative. The sole spot observed had an R_F value intermediate between those of maltotriose and laminaritriose, so that the trisaccharide appeared to contain one 1 : 4- and one 1 : 3-link per molecule. Confirmation of this was obtained when partial hydrolysis of the trisaccharide yielded 3-*O*-α-D-glucosyl-D-glucose, maltose, and D-glucose. It was to be expected that the trisaccharide fraction contained two components, with the structures shown in (I) and (II), and indeed this was proved



(III) Broken lines signify points of rupture by phenylhydrazine.

in two ways. First, partial acidic hydrolysis of the hypoiodite-oxidised trisaccharide fraction yielded, *inter alia*, maltose and 3-*O*-α-D-glucosyl-D-glucose. Secondly, in collaboration with Dr. A. B. Foster, the two trisaccharides were separated by paper ionophoresis of their borate complexes, their M_G values being compatible with the proposed structures (cf. Foster, *J.*, 1953, 982).

It thus appeared probable that a substantial proportion of the " mycodextran " mole-

cule contained α-D-glucopyranose units linked alternately through positions 3 and 4. Supplementary evidence in support of this conclusion was obtained when the periodate-oxidised polysaccharide (III) was treated with phenylhydrazine, a technique which has been used, for example, by Barry (*Nature*, 1943, **152**, 537) and by O'Colla (*Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 165) and is based on the fact that phenylhydrazine converts an acetal of glyoxal [$OHC{\cdot}CH(OR)_2$] into glyoxalosazone, with the liberation of the alcohol (ROH). From the mixture of osazones thus obtained, D-glucosazone was isolated in an overall yield from " mycodextran " of 22%; in the absence of any significant degree of branching, it must have arisen from glucose residues joined through positions 1 and 3 to two periodate-sensitive units. The absence of a polysaccharide osazone from the products eliminated any possibility that long sequences of α-1 : 3-linkages occur in " mycodextran."

" Mycodextran " bears a resemblance to Floridean starch ($[\alpha]_D$ +156° in H_2O), inasmuch as they both contain appreciable proportions of 1 : 3- and 1 : 4-glucosidic bonds (cf. Barry, Halsall, Hirst, and Jones, *J.*, 1949, 1468), but the former polysaccharide has a higher optical rotation, suggesting that a greater proportion of the linkages are in the α-form. It is now apparent that the " mycodextran " structure is markedly different from those of dextrans, in which the principal glucosidic linkages are of the α-1 : 6-type, so that the term " mycodextran " must inevitably lead to confusion. We suggest that the polysaccharide should now be known as " nigeran," and 3-*O*-α-D-glucopyranosyl-D-glucose as " nigerose."

Experimental

Isolation of " Mycodextran " (Nigeran).—The polysaccharide, kindly supplied by Dr. J. L. Yuill, had been isolated from *Aspergillus niger* (strain 152), which had been grown on sucrose and washed thoroughly with cold water. The mycelium had been extracted with boiling water for 5 min., and the filtered extract cooled to precipitate the polysaccharide. Sample (A) was the product from four successive extracts, whereas sample (B) was obtained from the third and fourth extracts. Both samples had been washed with alcohol and then with ether, before being dried at 55—60°.

Examination of Sample (B).—(a) *Reducing power.* The method employed was essentially that described by Meyer *et al.* (*loc. cit.*). The reagent—3 : 5-dinitrosalicylic acid (1·5 g.) in 0·2N-sodium hydroxide (100 c.c.)—had been calibrated against maltose hydrate. The reagent (1 c.c.) was added to a solution of sample (B) (0·10 g.) in 2N-sodium hydroxide (3 c.c.), and the mixture was kept at 65° for 30 min., cooled, and diluted with water (20 c.c.), before the intensity of colour was determined. In order to correct for the turbidity of the polysaccharide solution, an extra blank containing no added reagent was treated similarly. The results indicated that the degree of polymerisation was *ca.* 175.

(b) *Reducing sugars liberated during acidic hydrolysis.* (i) *Identification.* Sample (B) (20 mg.) was kept at 100° for 5 hr. with 2N-sulphuric acid (1 c.c.) in a sealed tube; the solution was diluted with water (2 c.c.), and freed from ions by means of an apparatus similar to that described by Consden, Gordon, and Martin (*Biochem. J.*, 1947, **41**, 590). The hydrolysate, together with reference solutions, was separated on paper chromatograms, irrigated with the upper phase of a mixture of *n*-butanol (40%), ethanol (10%), water (49%), and ammonia (1%), for 24 hr. (solvent front retained on the paper) and 48 hr., respectively. Only one sugar component was revealed on development with aniline hydrogen phthalate (Partridge, *loc. cit.*), and this had an R_F value identical with that of the glucose reference spot.

(ii) *Determination.* Sample (B) was hydrolysed for 6 hr. with 7% sulphuric acid at 100°, and the glucose produced was determined by cuprimetric titration, as described by Bourne, Donnison, Haworth, and Peat (*J.*, 1948, 1687). After a small correction (3%) had been applied for the loss in reducing power which occurs with glucose itself under these conditions (Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224), the reducing power of the neutral hydrolysate corresponded to a conversion into glucose of 97—98%.

(c) *Optical rotation.* The polysaccharide (B), although sparingly soluble in cold water, dissolved readily in hot water. It had $[\alpha]_D^{16}$ +283° (*c*, 0·78 in N-NaOH), compared with +270° (*c*, 1·04) for sample (A).

(d) *Iodine stain.* Sample (B) gave no detectable stain with iodine under the standard conditions for the determination of blue value (B.V.) described by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924).

Partial Acidic Hydrolysis of the Polysaccharide.—Sample (B) (1·363 g.) was partially hydrolysed with N-sulphuric acid (150 c.c.) at 85° for 3 hr. The hydrolysate then had $[\alpha]_D^{23}$ +121° (*c*, 0·68 in H_2O), and the reducing power corresponded to a 63·3% conversion into glucose. The saccharide mixture was fractionated on a charcoal column (17 × 3·4 cm.) by Whistler and Durso's method (*loc. cit.*). The column was washed as shown in the Table. Each of the eluates was neutralised with barium carbonate, concentrated to a small volume (*ca.* 25 c.c.), filtered, and freeze-dried to a white solid or a syrup, which was subsequently dried to constant weight at 60° in a vacuum over phosphoric oxide.

(a) D-*Glucose*. The product from the aqueous eluate, crystallised from methanol, gave α-D-glucose (0·365 g.), m. p. and mixed m. p. 139—142°, $[\alpha]_D^{17}$ +101·5° (2·5 min.) ⟶ +53·1° (equil.) (*c*, 0·85 in H_2O). From the mother-liquors D-glucosazone [m. p. and mixed m. p. 204—205° (decomp.)], D-glucophenylosotriazole (m. p. and mixed m. p. 195—197°), and β-D-glucose penta-acetate (m. p. and mixed m. p. 129—131°) were prepared.

(b) 3-O-α-D-*Glucopyranosyl*-D-*glucose* [*Disaccharide* (*X*)]. A duplicate experiment, carried out on a larger scale with sample (A), gave a 5% ethanol eluate rich in disaccharide (X), but contaminated with a trace of maltose. The freeze-dried solid (0·565 g.), phenylhydrazine (1·5 c.c.), glacial acetic acid (1·5 c.c.), and water (7·5 c.c.) were kept at 100° for 30 min., and cooled. Washed with water and crystallised from ethanol, the precipitate gave turanose phenylosazone (0·207 g.), m. p. and mixed m. p. 204—206° (Found: N, 11·0. Calc. for $C_{24}H_{32}O_9N_4$: N, 10·8%). Other samples of the osazone were prepared similarly.

An aqueous solution (9 c.c.) of the osazone (0·306 g.) was heated on a boiling-water bath for 30 min. with cupric sulphate pentahydrate (0·440 g.), cooled, filtered, saturated with hydrogen sulphide, and filtered again. The filtrate was neutralised with barium carbonate, clarified, and extracted with ether to remove the aniline. The aqueous layer was concentrated to a syrup, which was crystallised from ethanol to give turanose phenylosotriazole (0·077 g.), m. p. and mixed m. p. 190—191° (Found: C, 50·6; H, 6·0; N, 9·6. Calc. for $C_{18}H_{25}O_9N_3$: C, 50·6; H, 5·9; N, 9·8%). The osazone and the osotriazole gave infra-red spectra identical, over the range 700—960 cm.$^{-1}$, with those of authentic specimens.

Disaccharide (X) (0·041 g.) was heated with acetic anhydride (0·41 c.c.) and anhydrous sodium acetate (0·011 g.) at 100—110° for 70 min. Isolated in the usual way and crystallised from ethanol, the *acetate* (0·027 g.) had m. p. 111—113° (Found: C, 49·7; H, 5·65. $C_{28}H_{38}O_{19}$ requires C, 49·6; H, 5·6%).

A syrupy disaccharide fraction (0·470 g.), containing both maltose and disaccharide (X), treated with 4·3% methanolic hydrogen chloride (20 c.c.) at room temperature, showed $[\alpha]_D^{17}$ +126·4° (3 min.) ⟶ +89·8° (74 min.) ⟶ +85·8° (20 hr.), whereas, under the same conditions, maltose hydrate showed $[\alpha]_D^{17}$ +127·5° (3 min.) ⟶ +126·1° (74 min.) ⟶ +112·6° (19 hr.). Thus it was probable that disaccharide (X) carried a free hydroxyl group at position 4 of the reducing unit. It is likely that maltose and disaccharide (X) could be separated more efficiently by virtue of their markedly different rates of reaction with methanolic hydrogen chloride than on a charcoal column, and this possibility is being explored.

(c) *Maltose*. A disaccharide fraction (0·074 g.), relatively rich in maltose but contaminated with a trace of disaccharide (X), was kept with acetic anhydride (2 c.c.) in pyridine (2 c.c.) at room temperature for 24 hr., giving β-maltose octa-acetate (0·089 g.), m. p. and mixed m. p. 158—159°, $[\alpha]_D^{17}$ +64·6° (*c*, 0·42 in $CHCl_3$) (Found: C, 49·3; H, 5·7. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6%).

(d) *Trisaccharide fraction*. The material eluted by 15% ethanol after partial hydrolysis of sample (B), was examined, as its benzylamine derivative (Bayly and Bourne, *loc. cit.*), on a paper chromatogram. Only one spot was revealed; it did not arise from *iso*maltotriose, laminaritriose, maltotriose, or panose. It had an R_F value intermediate between those of the benzylamine derivatives of laminaritriose and maltotriose, which suggested that the trisaccharide fraction had a 1 : 3- and a 1 : 4-glucosidic bond. However, electrophoretic separation of the fraction, in borate buffer, on filter paper showed two components with mobilities similar to those of the α-1 : 3- and α-1 : 4-linked disaccharides (cf. Foster, *loc. cit.*).

Partial hydrolysis of the trisaccharide fraction (5 mg.) with N-sulphuric acid (1 c.c.) at 90° for 30 min. gave three products, having R_F values identical with those of glucose, maltose, and 3-*O*-α-D-glucopyranosyl-D-glucose. Both disaccharides, *inter alia*, were again evident in a hydrolysate of hypoiodite-oxidised trisaccharide.

Acetylation of Sample (B).—The polysaccharide (95·2 mg.), acetic anhydride (2 c.c.), and pyridine (2 c.c.) were kept at room temperature for 48 hr. and then at 100° for 3 hr. The *acetate* (140·5 mg.) was precipitated with water, washed, and dried over phosphoric oxide at

60°/15 mm.; it had $[\alpha]_D^{17}$ +157° (*c*, 0·29 in $CHCl_3$) (Found: C, 50·3; H, 5·7. A tri-*O*-acetyl-glucosan $C_{12}H_{16}O_8$ requires C, 50·0; H, 5·6%).

Methylation of Sample (B).—The dried polysaccharide (0·935 g.) was treated with sodium and methyl iodide in liquid ammonia at −70° (cf. Freudenberg and Boppel, *Ber.*, 1938, **71**, 2505). After four additions of the methylating reagents had been made, the ammonia was allowed to evaporate under anhydrous conditions, the last traces being removed at 55°/15 mm. Extraction of the residue with chloroform (Soxhlet) gave, from the extract, a solid (1·088 g.) having $[\alpha]_D^{17}$ +198·7° (*c*, 0·40 in $CHCl_3$) (Found: OMe, 39·5. Calc. for a tri-*O*-methylglucosan $C_9H_{16}O_5$: OMe, 45·6%). Part (0·857 g.) of this material was treated with four more portions of the methylating reagents, and, after further treatment as above, the product was dialysed against several changes of distilled water, and the water-soluble *ether* was freeze-dried to a white solid (overall yield, 64%), $[\alpha]_D^{17}$ +217·8° (*c*, 0·70 in $CHCl_3$) (Found: OMe, 45·0%).

Methylation of Sample (A).—The polysaccharide (6·86 g.), purified by "crystallisation" from water, was methylated as above, eight additions of the methylating reagents being made. The product was suspended in water (150 c.c.), and extracted with chloroform (250 c.c.; 50 c.c.; 50 c.c.). The combined extracts were washed with water, filtered, and evaporated, the residue being ground with ether and dried to a white powder, from which impurities soluble in light petroleum (b. p. 60—80°) were removed in a Soxhlet apparatus. The overall yield was 67% (Found: OMe, 44·6%).

Methanolysis and Hydrolysis of the Polysaccharide Ethers.—(a) *Paper chromatographic analysis of the* O-*methylglucoses.* The methyl ether of sample (B) was submitted to methanolysis and then to hydrolysis with 4% hydrochloric acid. A paper chromatogram of the hydrolysate, freed from ionised impurities as above, was irrigated for 48 hr. as before, and sprayed with aniline hydrogen phthalate; it showed two principal spots, of approximately equal intensity, having colours and R_F values identical with those of spots of 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methyl-glucose (brown and red stains, respectively). They were distinguished from 2 : 3 : 4- and 2 : 3 : 5-tri-*O*-methylglucose on the same chromatogram; no di-*O*-methylglucose could be detected.

A similar result was obtained with the methyl ether of sample (A). In addition, an intense spot corresponding in colour, and in R_F value, to 2 : 3 : 4 : 6-tetra-*O*-methylglucose was revealed after it had been enriched by chloroform–water partition.

(b) *End-group assay.* The methyl ether of sample (A) (0·488 g.) was treated for 7 hr. with boiling methanolic 8·7% hydrogen chloride (40 c.c.). The residue obtained when the solution was neutralised with lead carbonate, filtered, and evaporated, was hydrolysed with 4% hydro-chloric acid (40 c.c.) at 100° for 6 hr. The aqueous solution was neutralised with lead carbonate, filtered, and evaporated, and the *O*-methylglucoses were extracted exhaustively with ether. The extracts yielded a syrup, which was dissolved in water (10 c.c.) and extracted with water-washed chloroform (9 × 10 c.c.). The combined extracts were washed with water (90 c.c.) before being concentrated to a syrup, in which the tri- and tetra-*O*-methylglucoses were determined according to the paper chromatographic procedure described by Hirst, Hough, and Jones (*loc. cit.*). The factors 1·62 and 825 were applied to give, respectively, the amounts of "tetra" and "tri" present before the enrichment process, and in this way two determinations gave chain lengths of 300 and 360 anhydroglucose units. [In these calculations, the partition coefficient of 2 : 3 : 4 : 6-tetra-*O*-methylglucose was assigned the value determined by Mac-donald (*J. Amer. Chem. Soc.*, 1935, **57**, 771); it was assumed that 2 : 4 : 6-tri-*O*-methylglucose had the same partition coefficient as that determined for its 2 : 3 : 6-isomer by Macdonald.]

(c) *Characterisation of the tri-*O*-methylglucoses.* A solution of the methyl ether (1·722 g.) of sample (A) in methanolic 3% hydrogen chloride (100 c.c.) was kept at 80° for 10 hr., neutralised with lead carbonate, filtered, and evaporated. The glycosides were treated with aqueous 4% hydrochloric acid (85 c.c.) at 100° for 5 hr., and the solution was neutralised with lead carbonate, filtered, and evaporated. Crystallisation of the chloroform-soluble portion from ether gave needles of 2 : 4 : 6-tri-*O*-methyl-α-D-glucose (0·394 g.), m. p. and mixed m. p. 114·5—116·5°, $[\alpha]_D^{16}$ +105° (2·5 min.) ⟶ +72·5° (3 days) (*c*, 0·5 in H_2O), $[\alpha]_D^{16}$ +84·3° (1·5 min.) ⟶ +72·6° (7 days) (*c*, 0·4 in methanolic 2% hydrogen chloride) (Found: C, 48·6; H, 8·0. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·2%). Lake and Peat (*J.*, 1938, 1417) gave m. p. 115°, $[\alpha]_D^{20}$ +98·2° ⟶ +74·8 (equil.) in H_2O.

The ethereal mother-liquors yielded a syrup (1·024 g.), which was boiled for 3 hr. with aniline (0·50 c.c.) in ethanol (5 c.c.). Recrystallisation of the product from ether gave *N*-phenyl-2 : 4 : 6-tri-*O*-methyl-D-glucosylamine (0·220 g.), m. p. 160—162°, $[\alpha]_D^{17}$ −113·5° (3·5 min.) ⟶ −79° (24 hr.) (*c*, 0·45 in dry MeOH) (Found: C, 60·8; H, 8·0; N, 4·9. Calc. for $C_{15}H_{23}O_5N$: C, 60·6; H, 7·8; N, 4·7%). Granichstädten and Percival (*loc. cit.*) recorded m. p. 162—166°,

$[\alpha]_D^{15}$ $-113°$ (initially, in MeOH); Connell, Hirst, and Percival (*J.*, 1950, 3494) gave m. p. 163—165°, $[\alpha]_D^{15}$ $-81°$ (20 hr., in MeOH).

The residual syrupy aniline derivatives were treated for 2 hr. with 2N-sulphuric acid at 100° in a stream of carbon dioxide. The solution was neutralised with barium carbonate, filtered, and extracted with ether. The aqueous layer yielded a syrup (S), which was extracted with chloroform. The soluble portion, crystallised from ether, gave 2 : 3 : 6-tri-*O*-methyl-α-D-glucose (0·145 g.), m. p. and mixed m. p. 113—115°, $[\alpha]_D^{16}$ $+69°$ (init.) ⟶ $-33°$ (19 hr.) (*c*, 0·36 in methanolic 2% hydrogen chloride) (Found: C, 48·6; H, 8·3. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·2%). Granichstädten and Percival (*loc. cit.*) recorded $[\alpha]_D^{18}$ $+70°$ (init.) ⟶ $-32°$ (24 hr.) (in methanolic 2% hydrogen chloride). A second sample (0·670 g.) of the syrup (S) was treated with *p*-phenylazobenzoyl chloride (2·20 g.) in pyridine (12 c.c.), and the products were fractionated on a column of alumina, as described by Coleman *et al.* (*loc. cit.*), to give 2 : 3 : 6-tri-*O*-methyl-αβ-D-glucose 1 : 4-bis-*p*-phenylazobenzoate (0·295 g.), $[\alpha]_{6438}^{19}$ $+5·7°$ (*c*, 0·60 in $CHCl_3$). Coleman *et al.* (*loc. cit.*) gave $[\alpha]_{6438}^{25}$ $-1°$, $+1°$, $-5°$ (in $CHCl_3$) for samples prepared in this way.

(d) *Relative proportion of the tri-*O*-methylglucoses.* An unfractionated mixture of *O*-methylglucoses, prepared as above, showed $[\alpha]_D^{16}$ $+70·8°$ (4 min.) ⟶ $+22·6°$ (24 hr.) (*c*, 0·68 in methanolic 2% hydrogen chloride). The final value corresponded to that shown by a mixture of 2 : 3 : 6- and 2 : 4 : 6-tri-*O*-methyl-D-glucose containing 47% of the former ether (cf. Granichstädten and Percival, *loc. cit.*). [As a first approximation, the contribution of the small proportion ($<$1%) of the tetra-*O*-methyl-D-glucose in the hydrolysate can be neglected.]

Periodate Oxidation of Sample (B).—(a) *Periodate consumed.* A suspension of sample (B) (0·1132 g.) in 0·30M-sodium periodate (5·00 c.c.) was diluted to 250 c.c., and shaken at room temperature for several days, during which the polysaccharide gradually dissolved. Aliquot portions (25 c.c.) of the solution, and also of a blank from which the polysaccharide had been omitted, were removed at intervals, and analysed for periodate by Fleury and Lange's method (*J. Pharm. Chim.*, 1933, **17**, 107, 196). The periodate consumption, expressed in mols. per anhydroglucose unit, was: 0·26 (3·6 hr.), 0·36 (20 hr.), 0·38 (24 hr.), 0·40 (28 hr.), 0·47 (44 hr.), 0·48 (92 hr.).

(b) *Formic acid produced.* After 94 hr., a portion (50 c.c.) of the solution was treated with ethylene glycol (1 c.c.), and titrated to pH 6·6 (pH meter) against 0·01N-sodium hydroxide; the result indicated that the formic acid production was 0·030 mol. per anhydroglucose unit.

(c) *Hydrolysis of the oxidised polysaccharide.* After 94 hr., a portion (20 c.c.) of the solution containing the oxidised polysaccharide was dialysed, concentrated, and hydrolysed with 0·1N-sulphuric acid. The hydrolysate, freed from mineral matter, was examined on a paper chromatogram, and gave, *inter alia*, a spot having an R_F value identical with that of glucose.

(d) *Treatment of the oxidised polysaccharide with phenylhydrazine.* A solution of sample (B) (0·100 g.) and 0·3M-sodium periodate (5·00 c.c.) in water (245 c.c.) was shaken at room temperature for 4 days, dialysed for 3 days, concentrated, and freeze-dried to a white solid (0·093 g.), which was then heated at 100° for 45 min. with a mixture (2·8 c.c.) of phenylhydrazine, acetic acid, and water (2 : 2 : 5, by vol.). The yellow precipitate dissolved when alcohol (15 c.c.) was added, indicating the probable absence of a polysaccharide osazone. Graded addition of water to the alcoholic solution gave glucosazone (0·020 g.), m. p. and mixed m. p. 203—204°.

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