116. *Immunopolysaccharides. Part VIII.* Enzymic Synthesis of* 6-O-α-D-*Glucopyranosyl*-3-O-*methyl*-D-*glucose by* Betacoccus arabinosaceous.

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Examination of the homologous series of oligosaccharides produced by *B. arabinosaceous* (Birmingham) in a medium containing sucrose and 3-*O*-methyl-D-glucose has shown that they are formed by successive addition of glucosyl units in α-1 : 6-linkage to 3-*O*-methyl-D-glucose. This observation is of interest in connection with the mechanism of synthesis of the methylated polysaccharides which have been found in Nature.

There is now ample evidence [1] that when *B. arabinosaceous* is grown on media containing sucrose and certain simple sugars the dextransucrase present can utilise the sucrose as its substrate and each added sugar as a receptor in the production of oligosaccharides. The structures of some of these oligosaccharides have already been reported.[1,2,3] The present investigation, in which 3-*O*-methyl-D-glucose was used as a receptor molecule, was of interest because the organism was known to synthesise a dextran containing α-1 : 3-linkages [4] and because of the known occurrence of 3-*O*-methyl-sugars in Nature (*e.g.*, 3-*O*-methyl-D-galactose [5] has been isolated from the hydrolysis products of slippery elm mucilage).

Chromatographically pure di-, tri-, and tetra-saccharides were isolated from a culture of *B. arabinosaceous* grown on a mineral medium containing sucrose (2%) and 3-*O*-methyl-D-glucose (10%). Of the glucose available from the sucrose, 20% was converted into disaccharide, 8% into trisaccharide, and 4% into tetrasaccharide.

The approximate molecular size of the disaccharide was ascertained by oxidation with alkaline hypoiodite [6] and by ionophoresis of its *N*-benzylglycosylammonium ion [7] in formate buffer (pH 1·8). The presence of one methoxyl group in the disaccharide was confirmed by a methoxyl determination.[8] Acidic hydrolysis yielded components identical with glucose and 3-*O*-methylglucose on paper chromatography and ionophoresis.

An α-glycosidic linkage in the disaccharide was indicated by (*a*) the relatively high specific rotation (+121° equil.), (*b*) its resistance to almond β-glycosidase, and (*c*) the presence of infrared absorption [9] at 837 cm.$^{-1}$ (α-linkages) and its absence from 890 cm.$^{-1}$.

* Part VII, *J.*, 1957, 3536.

[1] Bailey, Barker, Bourne, and Stacey, *Nature*, 1955, **175**, 635; *J.*, 1957, 3536.
[2] *Idem*, *Nature*, 1955, **176**, 1164.
[3] Barker, Bourne, Grant, and Stacey, *Nature*, 1956, **178**, 1221.
[4] Barker, Bourne, Bruce, Neely, and Stacey, *J.*, 1954, 2395.
[5] Hirst, Hough, and Jones, *Nature*, 1950, **165**, 34; *J.*, 1951, 323.
[6] *Idem*, *J.*, 1949, 928.
[7] Barker, Bourne, Grant, and Stacey, *Nature*, 1956, **177**, 1125.
[8] Belcher, Fildes, and Nutten, *Analyt. Chim. Acta*, 1955, **33**, 16.
[9] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.

The high mobility of its borate complex [10] on ionophoresis was indicative of a 1 : 6- or 1 : 3-glycosidic linkage.

The disaccharide consumed 2·97 mol. of periodate and produced 1·0 mol. of formic acid, but no formaldehyde. These values do not correspond to any of the theoretical values for 3-*O*-methylglucosylglucose structures containing a 1 : 2-, 1 : 3-, 1 : 4-, or 1 : 6-glycosidic linkage. Of the various glucosyl-3-*O*-methylglucose structures the only candidates are those containing a 1 : 4 or 1 : 6-glycosidic linkage. The possibility of the former was excluded by the relatively high M_G value [10] of the disaccharide and by its ability to form a methyl furanoside. The assignment of the structure 6-*O*-α-D-glucopyranosyl-3-*O*-methyl-D-glucose to the disaccharide was confirmed by periodate oxidation of the methyl furanoside which consumed 1·8 mol. of periodate and produced 0·75 mol. of formic acid (theor.: 2 mol. of periodate, 1 mol. of formic acid). Hydrolysis of the periodate-oxidised methyl furanoside produced a component which moved similarly to 3-*O*-methylglucose on paper chromatographic and ionophoretic examination. Since 6-*O*-methylglucose resembles 3-*O*-methylglucose in its behaviour on paper chromatograms and paper ionophoretograms the above evidence is consistent with the disaccharide's being 3-*O*-α-D-glucopyranosyl-6-*O*-methylglucose, but this structure would involve transmethylation and is considered to be unlikely.

The molecular sizes of the trisaccharide and the tetrasaccharide were proved as for the disaccharide, and again total acidic hydrolysis gave only glucose and 3-*O*-methylglucose and the presence of one methoxyl group was confirmed as before. The products of partial acidic hydrolysis were in conformity with the structures *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-3-*O*-methyl-D-glucose and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-3-*O*-methyl-D-glucose.

The presence of α-linkages in both tri- and tetra-saccharide was confirmed by methods (*a*—*c*) above. The high mobilities of their borate complexes [10] were additional confirmation of 1 : 6-glycosidic linkages to their reducing units. Further evidence for the structure of the trisaccharide was obtained when it consumed 4·8 mol. of periodate with the production of 1·8 mol. of formic acid and no formaldehyde (theor.: 5, 2, 0 respectively).

The structures of the di-, tri-, and tetra-saccharide therefore appear to conform with their formation by successive addition of glucosyl units in α-1 : 6-linkage to 3-*O*-methyl-D-glucose, also with previous studies where glucose and methyl α-D-glucoside were used as glucosyl acceptors.

Experimental

Synthesis and Separation of the Oligosaccharides.—An aqueous medium (70 ml.) containing yeast extract (1%), $Na_2NH_4PO_4$ (0·5%), KH_2PO_4 (0·1%), $MgSO_4,7H_2O$ (0·05%), sucrose (2%), and 3-*O*-methyl-D-glucose (10%) was adjusted to pH 7 with sodium hydroxide and steam-sterilised at 15 lb./sq. in. for 30 min. This was then inoculated with *B. arabinosaceous* (Birmingham) and incubated at 25° for 3 days during which the pH fell to 4·6. After addition of ethanol (70 ml.), filtration, neutralisation, and removal of the ethanol, the residual solution was passed down a charcoal–" Celite " column [11] (l, 60 cm.; diam. 8 cm.). Washing with water eluted the monosaccharides and salts which were discarded. Gradient elution [12] (0 → 20% aqueous ethanol) then gave a mixed disaccharide fraction (375 mg.) and chromatographically pure trisaccharide (84 mg.) and tetrasaccharide (41·4 mg.) fractions. A small amount (20 mg.) of higher oligosaccharides was also obtained. Refractionation of the mixed disaccharide fraction on a charcoal–" Celite " column removed a ketose-containing disaccharide, which reacted with naphtharesorcinol,[13] and left a disaccharide (290 mg.) which showed a single component on paper chromatography.

Characterisation of the Disaccharide.—(i) The *disaccharide* (Found: OMe, 8·1. $C_{12}H_{21}O_{10}$·OMe

[10] Foster, *J.*, 1953, 982.
[11] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[12] Lindberg and Wickberg, *Acta Chem. Scand.*, 1954, **8**, 569.
[13] Forsyth, *Nature*, 1948, **161**, 239.

requires OMe, 8·7%) showed infrared absorption peaks at 968, 921, 863, 837, and 767 cm.$^{-1}$ in the range 700—1000 cm.$^{-1}$. It gave <0·5% ash and had $[\alpha]_D^{20}$ +121° (equil.) (*c* 0·915 in H_2O).

(ii) *Paper chromatography and ionophoresis.* On a paper chromatogram irrigated with the organic phase of a butanol–ethanol–water–ammonia mixture (40 : 10 : 49 : 1) the disaccharide moved as a single component with $R_{Glucose}$ 0·65. When separated as its benzylamine derivative [14] in the same solvent it had $R_{Glucose}$ 0·77. On paper ionophoresis [10] in borate buffer (pH 10) the mobility of the disaccharide was 0·54. When separated as its *N*-benzylglycosylammonium ion [7] in an electrolyte of sodium hydroxide–formic acid (pH 1·8), the mobility (*M*) was 0·70.

(iii) *Oxidation with hypoiodite.* The disaccharide (4·3 mg.), on oxidation with alkaline hypoiodite,[6] consumed iodine equivalent to 2·0 mg. of glucose (92% of the theoretical value for a glucosyl-mono-*O*-methylglucose).

(iv) *Hydrolysis.* The disaccharide (5 mg.) was heated with 1·5N-sulphuric acid (3 ml.) at 100° for 4 hr. Paper chromatography and ionophoresis of the neutralised hydrolysate indicated the presence of glucose and 3-*O*-methyl-D-glucose.

The disaccharide (5 mg.) was incubated at 37° with almond emulsin [20 mg. in 0·05M-acetate buffer (3 ml., pH 5)]. Paper chromatography of the digest after incubation for 3 days indicated only negligible hydrolysis to glucose and 3-*O*-methylglucose while controls containing cellobiose, lactose, or laminaribiose showed complete hydrolysis after the same time.

(v) *Periodate oxidation.* The disaccharide (76·2 mg.) was oxidised with 0·075M-sodium periodate (50 ml.) at 18°. The periodate consumption, expressed in moles per mole of disaccharide, was: 1·18 (0·5 hr.), 2·30 (1·0 hr.), 2·46 (3 hr.), 2·97 (24 hr.), and 2·97 (36 hr.). After 36 hr., 1·0 mole of formic acid, but no formaldehyde, had been produced per mole of disaccharide.

The disaccharide (1·2 mg.) was dissolved in 4% methanolic hydrogen chloride (4·8 ml.) and left at room temperature. It showed $[\alpha]_D^{20}$ +54° (5 min.) ⟶ +34° (45 min., equil.). After 45 min. the solution was filtered, neutralised with silver carbonate (*ca.* 2 g.), filtered, concentrated *in vacuo*, and then freeze-dried (61·2 mg.); $[\alpha]_D^{20}$ was +45·7° (*c* 1·0 in H_2O). This methyl furanoside (35·4 mg.) was oxidised with 0·02M-sodium periodate (25 ml.) at 18°. The periodate consumed, expressed in moles per mole of methylfuranoside, was: 0·70 (45 min.), 1·06 (3 hr.), 1·60 (24 hr.), 1·80 (72 hr.). After 72 hr., 0·75 mole of formic acid had been produced per mole of furanoside.

Ethylene glycol (0·2 ml.) was added to part (10 ml.) of the fully oxidised solution. After 10 min., 4·5N-sulphuric acid (5 ml.) was added and the solution heated at 100° for 1·5 hr. Free iodine was extracted with chloroform, and the aqueous solution neutralised with barium carbonate. On concentration and paper chromatography and ionophoresis the filtrate was found to contain a component chromatographically identical with 3-*O*-methyl-D-glucose.

Characterisation of the Tri- and Tetra-saccharides.—(i) The *trisaccharide* (Found: OMe, 5·8. $C_{18}H_{31}O_{15}OMe$ requires OMe, 6·0%) showed infrared absorption peaks at 901, 843, and 761 cm.$^{-1}$ in the range 700—950 cm.$^{-1}$. The *tetrasaccharide* (Found: OMe, 4·8. $C_{24}H_{41}O_{20}\cdot OMe$ requires OMe, 4·6%) showed infrared absorption peaks at 940, 919, 844, and 765 cm.$^{-1}$ in the same range. Both the trisaccharide {$[\alpha]_D^{20}$ +130° equil. (*c* 1·00 in H_2O)} and the tetrasaccharide fraction {$[\alpha]_D^{20}$ +129° equil. (*c* 1·00 in H_2O)} gave less than 0·5% of ash.

(ii) *Paper chromatography and ionophoresis.* On paper chromatography in the solvent used above both the tri- and the tetra-saccharide moved as single components with $R_{Glucose}$ 0·18 and 0·054, respectively. When separated as their benzylamine derivatives [14] in the same solvent they had $R_{Glucose}$ 0·35 and 0·15 respectively. The mobilities of the borate complexes [10] of the tri- and the tetra-saccharide were 0·49 and 0·42 while those of their *N*-benzylglycosylammonium ions were 0·58 and 0·47 respectively.

(iii) *Oxidation with hypoiodite.* On oxidation with alkaline hypoiodite,[6] the trisaccharide (3·6 mg.) consumed iodine equivalent to 1·30 mg. of glucose (104% of the theoretical value) while the tetrasaccharide (3·2 mg.) consumed iodine equivalent to 0·85 mg. of glucose (100%).

(iv) *Hydrolysis.* After hydrolysis as above with 1·5N-sulphuric acid at 100° for 4 hr., paper chromatography and ionophoresis showed that both the tri- and the tetra-saccharide gave only glucose and 3-*O*-methylglucose.

Portions (5 mg.) of tri- and tetra-saccharide were partially hydrolysed with N-sulphuric acid (3 ml.) at 90° for 1 hr. Paper chromatography and ionophoresis indicated that the partial

[14] Bayly and Bourne, *Nature*, 1953, **171**, 385.

hydrolysate of the trisaccharide contained glucose, 3-*O*-methylglucose, isomaltose, the disaccharide, and the original trisaccharide. The partial hydrolysate of the tetrasaccharide contained glucose, 3-*O*-methylglucose, isomaltose, the disaccharide, isomaltotriose, the trisaccharide and the original tetrasaccharide.

The trisaccharide and the tetrasaccharide were resistant when incubated with almond emulsin (as above).

(v) *Periodate oxidation.* The trisaccharide (15·7 mg.) was oxidised with 0·075M-sodium periodate (10 ml.) at 18°. The periodate consumption, expressed in moles per mole of trisaccharide, was 2·3 (0·5 hr.), 4·2 (1·0 hr.), 4·7 (3·0 hr.) and 4·8 (20 hr.). After 20 hr., 1·8 moles of formic acid had been produced per mole of trisaccharide.

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