219. *Biosynthesis of α-D-Glucopyranosyl D-Galactofuranoside and Other D-Galactose-containing Saccharides by* Betacoccus arabinosaceous.

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B. arabinosaceous grown on a galactose–sucrose medium produces di- and oligo-saccharides. One of the disaccharides has been characterised as α-D-glucopyranosyl D-galactofuranoside. The glucose : galactose ratios of the oligosaccharides suggest that they are formed by successive addition of glucosyl units to a galactose-containing receptor.

IT has been shown [1] that in cultures of *Betacoccus arabinosaceous* (Birmingham), containing sucrose as a substrate and isomaltose, maltose, methyl α-D-glucoside, or 3-*O*-methyl-D-glucose as receptor, oligosaccharides are formed by successive transfer of glucosyl units from the sucrose molecules to the receptor, with the formation of α-1,6-glycosidic linkages; with isomaltose and maltose the transfer was to the non-reducing moiety. In a previous paper [2] we established that the synthesis of the " branched " trisaccharide *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose in a lactose–sucrose medium proceeds by transfer of the glucosyl residue from sucrose to position 2 of the reducing unit of lactose. A similar trisaccharide, *O*-β-D-glucopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose is elaborated by the same organism when grown on a cellobiose–sucrose medium.[3] It was thought that the non-reducing moieties, β-D-galactosyl or β-D-glucosyl, in lactose and cellobiose did not provide suitable acceptor sites and thus tended to favour the transfer of the glucosyl residue from sucrose to the reducing moiety (αβ-D-glucose) of the receptor disaccharide. The present investigation, in which D-galactose was used as receptor, was of interest because the sugar was now present as α- and β-D-galactose and, moreover, for the preferred C1-conformation, its 4-hydroxyl group was axial, in contrast to the non-glucosidic hydroxyl groups of glucose.

A culture of *B. arabinosaceous*, grown on a D-galactose–sucrose medium, produced three disaccharides (A, B, and C) and two oligosaccharides (D and E). The sugars were isolated from the culture medium by fractionation on a charcoal–" Celite " column [4] and, when necessary, purified by paper chromatography. We now report the structure of disaccharide A and some properties of the other sugars.

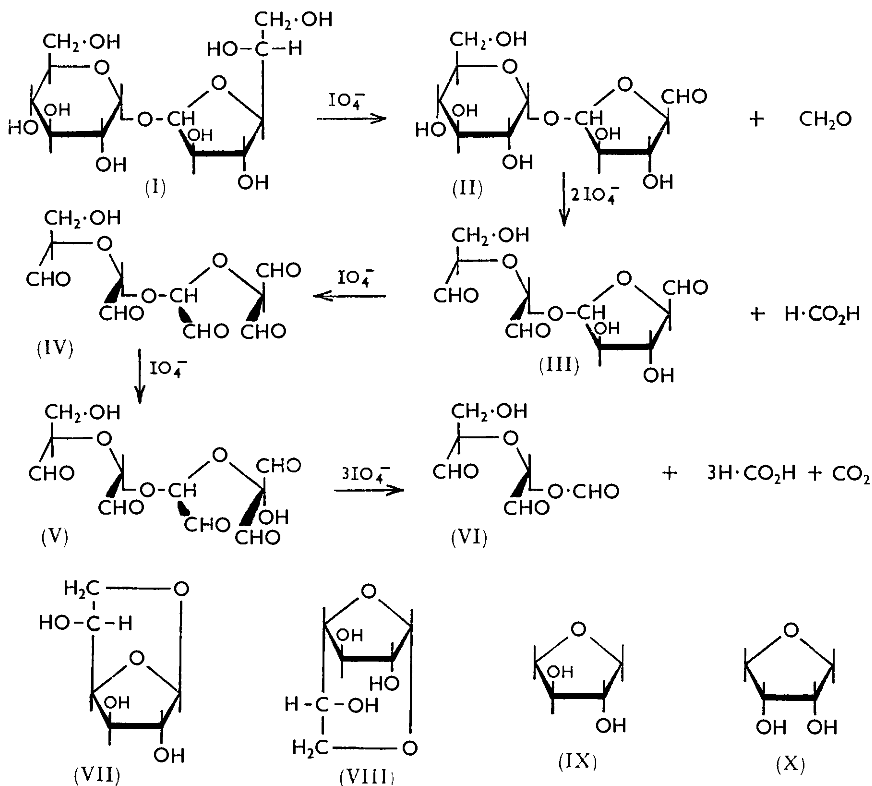
Chromatographically pure disaccharide A could be detected on paper chromatograms with acetone–silver nitrate–alcoholic sodium hydroxide,[5] but not with reagents specific for reducing sugars. Acid-hydrolysis yielded components identical with D-glucose and D-galactose on paper chromatography. Disaccharide A was immobile during electrophoresis in molybdate solution.[6] That it was a disaccharide was shown by the ratio of the constituent monosaccharides (glucose to galactose, 1 : 1·05) together with its rate of elution from a charcoal–" Celite " column and migration during chromatography.

Evidence for the non-reducing nature of disaccharide A was obtained by oxidation with alkaline hypoiodite [7] and Somogyi and Nelson's alkaline copper reagent,[8] and was confirmed when potassium borohydride failed to reduce it (paper chromatography of the product of an attempted reduction revealed a single component identical with disaccharide A; this component did not migrate during electrophoresis in molybdate solution, as would be expected of a disaccharide; [6] acid hydrolysis yielded only glucose and galactose).

[1] Bailey, Barker, Bourne, and Stacey, *J.*, 1957, 3530, 3536; Barker, Bourne, Grant, and Stacey, *J.*, 1958, 601.
[2] Bourne, Hartigan, and Weigel, *J.*, 1959, 2332.
[3] Bailey, Barker, Bourne, Grant, and Stacey, *J.*, 1958, 1895.
[4] (*a*) Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677; Lindberg and Wickberg, *Acta Chem. Scand.*, 1954, **8**, 569; (*b*) Barker, Bourne, and Theander, *J.*, 1955, 4276.
[5] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[6] Bourne, Hutson, and Weigel, *Chem. and Ind.*, 1959, 1047.
[7] Van der Plank, *Biochem. J.*, 1936, **30**, 457.
[8] Nelson, *J. Biol. Chem.*, 1944, **153**, 375; Somogyi, *ibid.*, 1945, **160**, 61.

Disaccharide A was hydrolysed by acid at a rate intermediate between those of αα-trehalose and sucrose and almost as fast as sucrose. This suggested that the glycosidic link between the D-glucosyl and the D-galactosyl unit was weakened by the presence of a furanosyl residue.

When treated with 5 mol. of periodate disaccharide A consumed in 800 min. 3·7 mol. of periodate and produced 1·45 mol. of formic acid and 0·83 mol. of formaldehyde. There was immediate consumption of 1 mol. of periodate followed by that of a further 2 mol. during the first 260 min. These values can be rationalised only when a D-glucopyranosyl D-galactofuranoside or a D-glucofuranosyl D-galactopyranoside structure is proposed for disaccharide A. Theoretically, complete oxidation of such a disaccharide (I) would consume 8 mol. of periodate and produce 4 mol. of formic acid, 1 mol. of formaldehyde, and 1 mol. of carbon dioxide (I—VI). The apparent discrepancy between the theoretical and the experimental values can be explained if the immediate oxidation stops with compound (III), whose further oxidation is then rate-determining. The resistance to periodate oxidation might be due to the formation of a cyclic semiacetal in the furanosyl



residue. Molecular models indicate that this is not so. On the other hand, 1,2-glycols derived from five-membered ring systems are not oxidised by periodate when the hydroxyl groups are securely locked in a *trans*-position, as in compounds (VII)[9] and (VIII):[10] L-threitan (IX), which has less securely locked *trans*-hydroxyl groups, reacts with periodate, but more slowly than the *cis*-compound erythritan (X);[11] further, Mitra and Perlin[12] have shown that the glucopyranosyl part of sucrose is more rapidly oxidised by periodate

[9] Dimler, Davis, and Hilbert, *J. Amer. Chem. Soc.*, 1946, **68**, 1377.
[10] Alexander, Dimler, and Mehltretter, *J. Amer. Chem. Soc.*, 1951, **73**, 4658.
[11] Klosterman and Smith, *J. Amer. Chem. Soc.*, 1952, **74**, 5336.
[12] Mitra and Perlin, *Canad. J. Chem.*, 1959, **37**, 2047.

than the fructofuranosyl part. On the basis of these facts we believe that the rate-determining step is the further oxidation of (III), the adjacent hydroxyl groups of which are *trans* to one another.* Thus in the later stages of the oxidation of disaccharide (I) the consumption of $(3 + 5x)$ mol. of periodate should give rise to $(1 + 3x)$ mol. of formic acid. The consumption of 3·7 mol. of periodate should, on this theory, produce 1·42 mol. of formic acid and 1·0 mol. of formaldehyde. The experimental results are in good agreement with these values and so, we suggest, prove the pyranosyl furanoside structure of disaccharide A.

That the initial attack of periodate on disaccharide A removed a primary hydroxyl group as formaldehyde was shown when use of 1 mol. of periodate produced 0·58 mol. of formaldehyde. The oxidation product was reduced with potassium borohydride to an acid-labile disaccharide. Paper chromatography of the hydrolysate revealed the presence of components corresponding to D-glucose and L-arabinose. This showed that disaccharide A was a D-glucopyranosyl D-galactofuranoside. D-Galactopyranosyl D-glucofuranoside should have yielded D-galactose and D-xylose.

The configuration of the glycosidic links was investigated by treatment of disaccharide A with hydrolytic enzymes. The disaccharide was hydrolysed by a mixture of yeast α-glucosidase and α-galactosidase, but not by the same mixture when the α-glucosidase was inhibited by the addition of D-glucono-δ-lactone.[13] It was not hydrolysed by almond β-glucosidase. We conclude that the D-glucopyranosyl unit had an α-configuration. There was not sufficient evidence to assign the configuration of the D-galactofuranosyl unit as it is likely that the α-galactosidase was specific for galactopyranosides.

The evidence presented thus characterises disaccharide A as α-D-glucopyranosyl D-galactofuranoside. This is of considerable interest since the synthesis of a furanoside was involved.

Disaccharides B and C could be detected on paper chromatograms with acetone–silver nitrate–alcoholic sodium hydroxide and with reagents specific for reducing sugars. Acid-hydrolysis of each yielded components identical with D-glucose and D-galactose on paper chromatography. They had a glucosyl-galactose structure as reduction with potassium borohydride followed by hydrolysis and paper chromatography revealed glucose and a hexitol.

The chromatographically pure oligosaccharides D and E yielded, when completely hydrolysed, components with R_F values identical with those of glucose and galactose in the ratios of 2 : 1 and 3 : 1, respectively. Partial hydrolyses gave components corresponding to glucose, galactose, and isomaltose on paper chromatograms. These oligosaccharides may thus be formed by successive addition of glucosyl units to a galactose-containing receptor molecule.

It is thus established that αβ-D-galactose serves as receptor of α-D-glucose units transferred from sucrose by *Betacoccus arabinosaceous*, though in view of the relatively low yields, much less efficiently than D-glucose.

Experimental

Chromatography.—The solvents used in chromatography were (*a*) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase); (*b*) butan-1-ol–benzene–pyridine–water (5 : 3 : 2 : 1); (*c*) butan-1-ol–pyridine–water–saturated aqueous boric acid (6 : 4 : 2 : 1).

Oligosaccharide Synthesis in a Galactose–Sucrose Medium.—An aqueous medium (150 ml.) containing yeast extract (1%), sodium ammonium hydrogen phosphate (0·5%), potassium dihydrogen phosphate (0·1%), hydrated magnesium sulphate (0·05%), and sucrose (2%) was adjusted to pH 7 with sodium hydroxide and sterilised, then inoculated with a strongly growing culture of *Betacoccus arabinosaceous* (Birmingham) and incubated at 25° for 60 hr.

* *Added*, 21.10.60: Since this paper was submitted, Kjølberg (*Acta Chem. Scand.*, 1960, **14**, 1118) has found that by lowering the temperature to 5° the periodate oxidation of methyl D-galactofuranosides and glucofuranosides can be confined to the 5,6-bond.

13 Conchie and Levvy, *Biochem. J.*, 1957, **65**, 389.

Galactose (20 g.), which had been purified by charcoal-column chromatography, was added and incubation continued for a further 5·5 days. Sterile 6% sucrose solutions (2·5 ml.) were added to the culture at intervals of 4 hr. throughout the first three days of the second incubation period.

The culture medium was adjusted to pH 7 and invertase (B.D.H. concentrate; 2 ml.) added to remove the residual sucrose. After incubation at room temperature for 1·5 hr. paper chromatography in solvent (*a*) and paper ionophoresis in borate buffer (pH 10) [14] revealed glucose, fructose, galactose, and oligosaccharides other than sucrose. The bacterial cells were removed by centrifugation (1 hr. at 5000 r.p.m.). After addition of ethanol (200 ml.) to precipitate dextran, the ethanol was removed from the supernatant liquid, and the residual solution was applied to a charcoal–" Celite " column [4] (30 × 8 cm.). The monosaccharides were eluted with water (4 l.), and the disaccharides with 5% aqueous ethanol (6 l.). Higher oligosaccharides were eluted with 10% (6 l.) and 15% aqueous ethanol (10 l.), respectively. Paper chromatography of the disaccharide fraction in solvent (*a*) showed the presence of one component with $R_{glucose}$ 0·46 and two components with $R_{glucose}$ *ca.* 0·38; ionophoresis in borate buffer (pH 10) revealed the presence of three components with M_G 0·54, 0·43, and 0·34, respectively.

Partial fractionation of the mixture of disaccharides was on a charcoal–" Celite " column (41 × 2·5 cm.) impregnated with 0·1M-borate buffer (pH 10), by elution with 0·1M-borate buffer (2·5 l.) and then with borate buffer containing 2·5% of ethanol (3 l.).[4b] Refractionation by paper chromatography in solvent (*a*) gave disaccharide A (47·6 mg.), B (32·7 mg.), and C (40·5 mg.).

Oligosaccharides D and E were obtained as chromatographically pure fractions when the charcoal–" Celite " column was eluted with 10% and 15% aqueous ethanol, respectively.

Properties of Disaccharide A.—(i) On a paper chromatogram irrigated with solvent (*a*) the disaccharide moved as single component with $R_{glucose}$ 0·46. Paper ionophoresis in borate buffer (pH 10) [14] again showed a single component with M_G 0·44. The disaccharide was revealed by acetone–silver nitrate–alcoholic sodium hydroxide [5] as a weak spot that appeared slowly and at the same rate as that due to αα-trehalose. It was not revealed with aniline hydrogen phthalate,[15] *p*-anisidine hydrochloride,[16] or triphenyltetrazolium chloride.[17]

The carbohydrate content, determined by the anthrone method,[18] was 96·1%.

(ii) Disaccharide A (*ca.* 1·5 mg.) was hydrolysed in 0·25N-sulphuric acid (2 ml.) at 100° for 40 min. Paper chromatography of the hydrolysate in solvent (*a*) showed components corresponding to glucose and galactose in equal quantities. The two components were isolated by paper chromatography. Determination of the glucose and galactose [8] in aliquot parts of the eluates revealed that they were present in the ratio of 1 : 1·05.

(iii) Disaccharide A (2·2 mg.), on oxidation with alkaline hypoiodite,[7] consumed iodine equivalent to 0·078 mg. of glucose or galactose (6·7% of the theoretical value for a disaccharide). When treated by the method of Somogyi and Nelson [8] disaccharide A had no reducing power.

(iv) Disaccharide A (2 mg.) was left in 0·05% potassium borohydride solution (0·5 ml.) overnight at room temperature. The solution was de-ionised by Amberlite resin IR-120 (H^+), then evaporated to dryness and distilled with dry methanol (3 × 2 ml.). Paper chromatography of the residue in solvents (*a*) and (*b*) revealed a single component identical with disaccharide A, immobile during electrophoresis in molybdate solution.[6]

This residue was hydrolysed with 1·5N-sulphuric acid at 100° for 4 hr. Paper chromatography of the de-ionised hydrolysate in solvent (*c*) revealed components corresponding to glucose and galactose.

(v) Disaccharide A (0·4 mg.), αα-trehalose (*ca.* 2 mg.), and sucrose (*ca.* 2 mg.) were separately hydrolysed with 0·25N-sulphuric acid (0·25 ml. and 1 ml.) at 100° for 15 min. Paper chromatography of the de-ionised hydrolysates showed that disaccharide A had been almost completely hydrolysed (*ca.* 90%) to glucose and galactose. Sucrose had been completely hydrolysed to glucose and fructose. Only a trace of glucose ($<10\%$) was liberated from αα-trehalose.

(vi) *Periodate oxidation.* Treatment of disaccharide A (10·3 mg.) with 0·015M-sodium

[14] Foster, *J.*, 1953, 982.
[15] Partridge, *Nature*, 1949, **164**, 443.
[16] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[17] Feingold, Avigad, and Hestrin, *Biochem. J.*, 1956, **64**, 351.
[18] Bailey, *Biochem. J.*, 1958, **68**, 669.

metaperiodate (10 ml.) in the dark at room temperature gave the following results. The periodate consumption, expressed in moles per mole of disaccharide A, was 1·2 (10 min.), 2·5 (150 min.), 2·9 (260 min.), 3·3 (480 min.), 3·5 (700 min.), 3·7 (800 min.). After 24 hr. all the periodate had been reduced. After 800 min. 1·45 moles of formic acid and 0·83 mole of formaldehyde were produced per mole of disaccharide A.

On treatment of αα-trehalose under the same conditions, the periodate consumption was 0·7 (10 min.), 3·2 (240 min.), 3·5 (360 min.), 3·6 (540 min.), 3·6 (1380 min.). After 540 min. 1·4 moles of formic acid were produced per mole of αα-trehalose. No formaldehyde was produced.

When the disaccharide A (0·34 mg.), dissolved in water (10 ml.), was treated with 0·001M-sodium metaperiodate (1 ml.) the formaldehyde produced (mole per mole) was 0·36 (7 hr.), 0·58 (23 hr.), 0·58 (48 hr.). The solution was then de-ionised with Permutit " Biodeminrolit " resin, pretreated with carbon dioxide. Potassium borohydride (50 mg.) in water (1 ml.) was added and the solution left overnight at room temperature, de-ionised by Amberlite resin IR-120 (H^+), and evaporated to dryness. The residue was distilled with dry methanol (3 × 2 ml.), then treated with 0·25N-sulphuric acid (5 ml.) at 100° for 15 min. The de-ionised hydrolysate was analysed by paper chromatography in solvent (*b*) which revealed components corresponding to D-glucose and L-arabinose, but not to galactose and xylose. They were detected with acetone–silver nitrate–alcoholic sodium hydroxide [5] and *p*-anisidine hydrochloride.[16] The latter reagent gave a pink stain, typical of pentoses with the component corresponding to L-arabinose and a yellowish stain with that corresponding to D-glucose.

(vii) A solution of disaccharide A (1%) was sealed in a capillary tube with an equal volume of almond β-glycosidase solution.[19] Paper chromatography of the digest after incubation at 35° for 72 hr. showed that no hydrolysis had occurred. Under the same conditions cellobiose was completely hydrolysed, lactose partially hydrolysed, and maltose was not hydrolysed. A 1% solution of disaccharide A was sealed in a capillary tube with an equal volume of yeast α-glucosidase [20] solution. Paper chromatography of the digest after incubation at 26° for 48 hr. revealed components corresponding to glucose and galactose. Under the same conditions, maltose was completely hydrolysed and melibiose partially hydrolysed. Lactose and cellobiose were not hydrolysed. A 1% solution of disaccharide A was sealed in a capillary tube with an equal volume of yeast α-glycosidase [20] solution, to which D-glucono-δ-lactone (2%) had been added: [13] paper chromatography of the digest after incubation at 26° for 72 hr. revealed absence of hydrolysis. Under the same conditions maltose was not hydrolysed and melibiose was partially hydrolysed.

Investigation of Disaccharides B and C.—(i) On a paper chromatogram irrigated with solvent (*a*) the disaccharides B and C moved with $R_{glucose}$ 0·39 and 0·36, and M_G 0·60 and 0·34, respectively. Disaccharide B was probably contaminated with a trace of leucrose. For detection see p. 1090.

(ii) Materials B (1 mg.) and C (1 mg.) in N-sulphuric acid at 100° for 4 hr. gave glucose and galactose (identified by paper chromatography). Sugar B also gave a trace of fructose.

(iii) Disaccharides B (2 mg.) and C (2 mg.) were separately treated with potassium borohydride (as above). The reduction products were hydrolysed with 1·5N-sulphuric acid at 100° for 4 hr. Paper chromatography of the hydrolysates in solvent (*c*) revealed components corresponding to glucose and a hexitol, but not galactose.

Investigation of Oligosaccharides D and E.—Chromatographically pure oligosaccharides D and E [$R_{glucose}$ 0·16 and 0·18, respectively, in solvent (*a*)] were separately hydrolysed in 1·5N-sulphuric acid at 100° for 4 hr. Paper chromatography then revealed components corresponding to glucose and galactose in the ratios of 2 : 1 and 3 : 1, respectively. Partial hydrolysis of D and E in N-sulphuric acid at 90° gave glucose, galactose, and isomaltose on paper chromatography.

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