385. *Immunopolysaccharides. Part IX.* The Enzymic Synthesis of Trisaccharides containing the α-1 : 2-Glucosidic Linkage.*

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A trisaccharide produced during the growth of *B. arabinosaceous* on a cellobiose–sucrose medium has been characterised as *O*-β-D-glucopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose. A similar trisaccharide elaborated in a lactose–sucrose medium has been characterised as *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.

In previous papers in this series [1,2] it has been shown that the primary function of the dextransucrase of *Betacoccus arabinosaceous* (Birmingham) is the transfer of a glucosyl moiety from its specific substrate sucrose to some suitable receptor (isomaltose, D-glucose, methyl α-D-glucoside, 3-*O*-methyl-D-glucose, or a growing dextran chain) to which it becomes attached by an α-1 : 6-glucosidic linkage. It was envisaged that under certain conditions the presence of an unsuitable non-reducing moiety (*e.g.*, β-D-glucosyl or β-D-galactosyl) in a receptor disaccharide might tend to favour the attachment of the enzymically transferred glucose residue to the reducing moiety of the disaccharide, if this has an appropriate structure. Such a transfer has indeed been found to occur (for preliminary reports see Bailey, Barker, Bourne, and Stacey; [3] Barker, Bourne, Grant, and Stacey [4]) where the receptor disaccharides were cellobiose and lactose. We now report fully the structural characterisation of the two trisaccharides thus formed.

Chromatographically pure trisaccharide A (in a yield corresponding to 11% of the glucose available from the sucrose) was isolated from a sucrose–cellobiose culture medium by fractionation on a charcoal–Celite column.[5,6] When separated as its *N*-benzylglycosylammonium ion, compound A had the mobility expected [7] of a trisaccharide. Its molecular size was confirmed by oxidation with alkaline hypoiodite.[8] On total acidic hydrolysis only glucose (98% conversion) was detectable by paper chromatography or ionophoresis. Partial hydrolysis yielded glucose, cellobiose, and a disaccharide X.

The reducing power of trisaccharide A with the Shaffer–Hartmann copper reagent [9] was only 7·7% of the theoretical value for a trisaccharide. This discrepancy is shown generally by 2-*O*-substituted glucoses.[3,8] The compound was not disclosed by alkaline triphenyltetrazolium chloride which detects all reducing glucosaccharides except those with a 2-*O*-substituent.[10] Further evidence that the reducing unit was linked through position 2 was obtained when treatment with phenylhydrazine gave a mixture of cellobiosazone and glucosazone. The low mobility (M_G 0·21) of the compound A on ionophoresis in borate buffer [11] was also indicative of a 1 : 2-linkage at the reducing group of the molecule.

Comparison of the specific rotation (+93°) of trisaccharide A with those of cellobiose (+35°) and maltotriose (+160°) suggested that one of the glycosidic linkages in the trisaccharide was α and the other β. Other evidence which confirmed this was (*a*) the

* Part VIII, *J.*, 1958, 601.

[1] Bailey, Barker, Bourne, and Stacey, *J.*, 1957, 3530.
[2] Barker, Bourne, Grant, and Stacey, *J.*, 1957, 3536.
[3] Bailey, Barker, Bourne, and Stacey, *Nature*, 1955, **176**, 1164.
[4] Barker, Bourne, Grant, and Stacey, *ibid.*, 1956, **178**, 1221.
[5] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[6] Lindberg and Wickberg, *Acta Chem. Scand.*, 1954, **8**, 569.
[7] Barker, Bourne, Grant, and Stacey, *J.*, 1957, 2067.
[8] Hirst, Hough, and Jones, *J.*, 1949, 928.
[9] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, **45**, 377.
[10] Feingold, Avigad, and Hestrin, *Biochem. J.*, 1956, **64**, 351.
[11] Foster, *J.*, 1953, 982.

presence of a peak at 840 cm.$^{-1}$ (α-linkage [12]) in the infrared spectrum (see Table) in addition to the peak at 894 cm.$^{-1}$ (β-linkage [12]) also shown by cellobiose, and (*b*) the action of almond β-glycosidase which produced glucose and disaccharide X. Disaccharide X, produced in this way, was shown to contain an α-glycosidic linkage by (*a*) its high specific rotation (+133°) and (*b*) the presence of a peak at 840 cm.$^{-1}$ in its infrared spectrum.

Disaccharide X was further characterised as 2-*O*-α-D-glucopyranosyl-D-glucose by (i) its low mobility on ionophoresis in borate buffer, (ii) its hydrolysis to glucose only (96% conversion), (iii) its low reducing power (27·5% of that calculated for a disaccharide) with Shaffer–Hartmann reagent,[9] (iv) its failure to react with alkaline triphenyltetrazolium chloride,[10] and (v) its stoicheiometric reaction with alkaline hypoiodite.[8] It was identical in its behaviour on paper chromatography and paper ionophoresis with a specimen of 2-*O*-α-D-glucopyranosyl-D-glucose synthesised chemically by Haq and Whelan.[13]

Infrared spectra (cm.$^{-1}$) of trisaccharides A and B, together with those of reference oligosaccharides.

Trisaccharide A	914	894	—	—	840	780
Cellobiose	914	892	—	—	—	778
Disaccharide X	913	—	—	—	840	780
Trisaccharide B	914	889	872	851	844	780
Lactose	913	896	872	—	—	780
Disaccharide Y	913	—	—	—	840	780
2-*O*-α-D-Glucosyl-D-glucose	913	—	—	—	840	780

From the evidence presented so far there are two possible structures for trisaccharide A, namely, (I) the "linear" *O*-β-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 2)-D-glucose and (II) the "branched" *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-D-glucopyranosyl-(1 → 2)]-D-glucose. From its mode of synthesis and the results of periodate oxidation of trisaccharide A [3·9 mols. of periodate consumed and 1·75 mols. of formic acid produced, leaving a fragment containing a glucose unit: theoretical values for (II) 4 mols. of periodate consumed and 2 mols. of formic acid produced] the structure of trisaccharide A was probably (II). This was confirmed by the results of periodate oxidation of the alcohol prepared from trisaccharide A by reduction with sodium borohydride. Trisaccharide A alcohol consumed 5·02 mols. of periodate and produced 1·92 mols. of formic acid and 0·85 mol. of formaldehyde, together with a product containing a xylose unit. [Theoretical for alcohols derived from (I) 6, 3, 1, and from (II) 5, 2, 1 respectively.] In addition, the alcohol consumed 5·2 mols. of lead tetra-acetate; only one of these was taken up rapidly, in conformity with structure (II), but not with (I).

Acidic hydrolysis of trisaccharide A alcohol resulted in 61·3% conversion into glucose; the other product of the hydrolysis was glucitol. On partial hydrolysis the products were glucose, glucitol, cellobiitol, and the alcohol derived from disaccharide X. The fact that two disaccharide alcohols were detected is further evidence in favour of structure (II).

Conclusive in favour of structure (II) were the products of hydrolysis of methylated trisaccharide A. One of the two methyl sugars appearing on a chromatogram was shown to be 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose by crystallisation of the free sugar and its anilide. The other was a di-*O*-methylhexose identical in its paper-chromatographic and -ionophoretic behaviour and in its infrared spectrum with 3 : 6-di-*O*-methyl-D-glucose.

Trisaccharide B was isolated, from a culture of *Betacoccus arabinosaceous* grown on a lactose–sucrose medium, in a yield corresponding to 31% of the glucose available from the sucrose. The structure of the trisaccharide was determined in a similar manner to that for trisaccharide A.

Compound B was shown to be a trisaccharide by the mobility of its *N*-benzylglycosylammonium ion [7] and by its almost stoicheiometric (96%) oxidation by alkaline hypoiodite.[8]

[12] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.
[13] Haq and Whelan, *Nature*, 1956, **178**, 1221.

On total acidic hydrolysis, both glucose and galactose were produced (ratio approx. 2 : 1). Partial hydrolysis gave glucose, galactose, lactose, an unknown disaccharide Y, and residual trisaccharide B.

The reducing power with the Shaffer–Hartmann copper reagent [9] was only *ca.* 6·8% of the theoretical value for a trisaccharide. Trisaccharide B did not react with alkaline triphenyltetrazolium chloride [10] and on treatment with phenylhydrazine gave a mixture of lactosazone and glucosazone. These properties together with the low mobility in borate buffer [11] were all indications that the reducing unit of trisaccharide B was linked through position 2.

As with trisaccharide A there was ample evidence that one of the glycosidic linkages in trisaccharide B was α and the other β. Trisaccharide B had a high specific rotation (+103°) compared with that of lactose (+52°), showed a peak at both 889 cm.$^{-1}$ (β-linkage) and 844 cm.$^{-1}$ (α-linkage) and was converted by almond β-glycosidase into galactose and disaccharide Y. Disaccharide Y showed properties identical with those of disaccharide X and 2-*O*-α-D-glucopyranosyl-D-glucose in all respects. Additional experiments carried out with disaccharide Y were (i) its treatment with phenylhydrazine to yield D-glucosazone and (ii) periodate oxidation of its alcohol (4·9 mols. of periodate consumed, 2·8 mols. of formic acid and 0·9 mol. of formaldehyde produced; theor. for a 1 : 2-linked disaccharide alcohol are 5, 3, 1 respectively). With both disaccharide X and disaccharide Y, periodate oxidation of the free sugar resulted in over-oxidation, perhaps due to a malondialdehyde structure's being produced during oxidation.

Partial hydrolysis of the alcohol derived from trisaccharide B yielded two disaccharide alcohols; this indicated a "branched" structure similar to that of trisaccharide A. Trisaccharide B consumed 3·9 mols. of periodate with the production of 1·75 mols. of formic acid but no formaldehyde (theor. for a "branched" structure are 4, 2, and 0 respectively). Trisaccharide B alcohol consumed 4·9 mols. of periodate with the production of 1·91 mols. of formic acid and 0·85 mol. of formaldehyde (theor. 5, 2, and 1 respectively). Hydrolysis of the periodate-oxidised trisaccharide and its alcohol yielded components corresponding to glucose and xylose, respectively. Thus it was apparent that $C_{(2)}$ and $C_{(4)}$ of the reducing glucose unit were engaged in glycosidic linkages. This was supported by the initial rapid uptake of 1 mol. of lead tetra-acetate by trisaccharide B alcohol.

Conclusive for the structure *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucopyranose were the products of hydrolysis of methylated trisaccharide B. Three spots were found on a chromatogram, characterised as those of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose, and 3 : 6-di-*O*-D-glucose.

The formation of branched trisaccharides by *B. arabinosaceous* is interesting as it involves the use of a carbohydrate primer in which a sugar residue, other than the usual non-reducing end unit, has the correct structure to act as an acceptor of a transferred sugar residue. This may be a general method for the formation of branched oligosaccharides and a possible route to the synthesis of branched polysaccharides which does not involve branching of a linear polymer as is the case with glycogen and amylopectin. It is interesting that the syntheses of trisaccharides A and B by the growing organism are accomplished by the formation in each case of an α-1 : 2-linkage and not the α-1 : 6-linkage usually produced by dextransucrase. Similar conversions have been effected by dextransucrase preparations, but the possibility that they are due to a second enzyme cannot be excluded. If, however, dextransucrase is responsible then it appears to be diverted from its preferred course of synthesis by the glycosidic substituent at position 4 of the receptor unit. This possibility will be examined further.

Cleavage of the β-linkages in trisaccharides A and B by almond β-glycosidase was extremely slow and in each case some 50% of the trisaccharide remained after 3 weeks. On the other hand the β-linkages of the two alcohols derived from the trisaccharides were completely hydrolysed in three days under comparable conditions. The reason for this marked difference undoubtedly lies in the fact that shielding of the β-linkage by the other

non-reducing sugar ring would be far greater in each of the trisaccharides than in the alcohols, the central unit of which has a more flexible open-chain structure.

Experimental

Oligosaccharide Synthesis in a Cellobiose–Sucrose Medium.—A sterile medium (100 ml.) was prepared containing yeast extract (1%), disodium ammonium phosphate (0·5%), potassium dihydrogen phosphate (0·1%), hydrated magnesium sulphate (0·05%) sucrose (10%), and cellobiose (30%). The medium was inoculated with *Betacoccus arabinosaceous* (Birmingham) and incubated at 25° for 3 days during which the pH fell from 7·0 to 4·1. Ethanol (100 ml.) was added, and the solution filtered and then adjusted to pH 7·0. The oligosaccharide mixture was fractionated [5,6] on a charcoal column (*l*, 60 cm.; diam. 8 cm.), the mono- and the di-saccharide fractions were discarded, and a trisaccharide fraction A (1·56 g.) was obtained by elution with 12·5% aqueous ethanol (8 l.).

Characterisation of Trisaccharide Fraction A.—(i) Trisaccharide A had $[\alpha]_D^{18}$ +93° (equil.; *c* 1·00 in H_2O) and gave <0·5% of ash. Paper chromatography of the free sugar and of its benzylamine derivative [14] in the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) showed that each moved as a single component with $R_{glucose}$ 0·14 and 0·32, respectively. The free sugar was detectable by aniline hydrogen phthalate [15] but not by alkaline triphenyltetrazolium chloride.[10] Paper ionophoresis [11] in borate buffer of pH 10 showed a single component (M_G 0·21). When separated as its *N*-benzylglycosylammonium ion [7] in an electrolyte of sodium hydroxide–formic acid (pH 1·8) the mobility (*M*) was 0·54.

(ii) *Oxidation by hypoiodite and copper reducing value.* Under conditions in which glucose was oxidised stoicheiometrically by iodine in alkaline solution (Hirst, Hough, and Jones [8]), trisaccharide A (10 mg.) consumed iodine equivalent to 3·75 mg. of glucose (105% of the expected theoretical value for a trisaccharide).

When estimated by the Shaffer–Hartmann method,[9] trisaccharide A (20 mg. and 10 mg.) had a reducing power equivalent to 0·54 mg. and 0·28 mg. of glucose, respectively. This corresponded to 7·6 and 7·8%, respectively, of the calculated value for a trisaccharide.

(iii) *Hydrolysis.* Complete hydrolysis of trisaccharide A (1·95 mg.) in 1·5N-sulphuric acid (3 ml.) at 100° for 4 hr. gave glucose (identified on a paper chromatogram and ionophoretogram), equivalent to a 98% conversion. Paper chromatography of a partial hydrolysate (N-sulphuric acid for 1 hr. at 90°) of trisaccharide A showed components corresponding to glucose, cellobiose, trisaccharide A, and an unknown disaccharide X ($R_{glucose}$ 0·44). The presence of these components was confirmed by ionophoresis in borate buffer, pH 10; the disaccharide X had M_G 0·32.

(iv) *Periodate oxidation.* Treatment of trisaccharide A (50·4 mg.) with 0·075M-sodium periodate (50 ml.) in the dark at room temperature gave the following results: The periodate consumption, expressed in moles per mole of trisaccharide A, was 1·0 (5 min.), 2·0 (10 min.), 2·7 (20 min.), 3·3 (60 min.), 3·9 (300 min.), 3·9 (1080 min.). After 1080 min., 1·75 moles of formic acid were produced per mole of trisaccharide A. No formaldehyde was produced.

Ethylene glycol (0·2 ml.) was added to part (10 ml.) of the fully oxidised solution. After removal of ions in a Consden desalting apparatus,[16] 4·5N-sulphuric acid (5 ml.) was added and the solution heated for 3 hr. at 100°. Paper-chromatographic and ionophoretic analysis of the neutralised hydrolysate indicated the presence of glucose.

(v) *Treatment with phenylhydrazine.* Trisaccharide A (0·10 g. in 1·7 ml.), when treated with phenylhydrazine (0·21 g.) and 50% acetic acid (0·21 ml.) at 100° for 2 hr., gave a mixture of osazones which was recrystallised twice from ethanol. Microscopical examination indicated the presence of cellobiosazone and glucosazone. The osazone mixture moved similarly to an authentic mixture of cellobiosazone and glucosazone when separated by paper ionophoresis in formate buffer of pH 1·8.

(vi) *Reduction of trisaccharide A.* Trisaccharide A (0·10 g.) in water (50 ml.) was reduced with sodium borohydride (0·11 g.) at room temperature for 2 hr. Amberlite IR-120 (H^+) (4 g.) was added, and the mixture shaken for 15 min., then filtered and evaporated to dryness *in*

[14] Bayly and Bourne, *Nature*, 1953, **171**, 385.
[15] Partridge, *Nature*, 1949, **164**, 443.
[16] Consden, Gordon, and Martin, *Biochem. J.*, 1947, **41**, 590.

vacuo. Dry methanol (3 × 25 ml.) was added and the whole evaporated to dryness *in vacuo*. The residue was dissolved in water and freeze-dried (73 mg.). The reduced trisaccharide A had $[\alpha]_D^{18}$ +89° (equil.; *c* 1·00 in H_2O) and moved as a single component ($R_{glucose}$ 0·19) in the solvent described above. It was not oxidised by alkaline hypoiodite [8] and had a negligible reducing power when determined by the Shaffer–Hartmann method.[9] The reducing sugars present in a complete hydrolysate (prepared as above) of trisaccharide A alcohol corresponded to 92% of the theoretical value. Paper chromatography of the hydrolysate and selective spraying with aniline hydrogen phthalate [15] and buffered bromocresol-purple revealed the presence of components having R_F values identical with those of glucose and glucitol. Similar analysis of a partial hydrolysate (0·2N-sulphuric acid at 100° for 1 hr.) showed the presence of glucose, glucitol, cellobiitol, trisaccharide A alcohol, and the alcohol derived from disaccharide X.

Treatment of trisaccharide A alcohol (21·2 mg.) with sodium periodate as above gave the following results. The periodate consumption, expressed in moles per mole of trisaccharide A alcohol, was: 3·25 (15 min.), 4·48 (60 min.), 4·80 (120 min.), 4·92 (180 min.), 5·02 (1200 min.). After 1200 min., 1·92 moles of formic acid and 0·85 mole of formaldehyde were formed per mole of trisaccharide A alcohol. Desalting [16] and acidic hydrolysis of the fully oxidised reaction solution (as above) gave a component similar to a pentose on paper-chromatographic analysis and identical with L-xylose on paper-ionophoretic examination.

Treatment of trisaccharide A alcohol (10 mg.) with almond β-glycosidase (1·5 ml.) at 37° for 3 hr. gave a solution which, by paper chromatography, was found to contain glucose and disaccharide X alcohol. After 3 days, no trisaccharide A alcohol remained.

Treatment of trisaccharide A alcohol (8·5 mg.) in glacial acetic acid (23 ml.) with a saturated solution of lead tetra-acetate (2 ml.) consumed the following no. of moles per mole of trisaccharide A alcohol: 1·3 (15 min.), 1·3 (60 min.), 1·9 (180 min.), 3·2 (1080 min.), 5·2 (after 60 min. at 45°).

(vii) *Methylation*. Trisaccharide A (0·2 g.) was suspended in dioxan (20 ml.) and methylated with dimethyl sulphate (15 ml.) and 30% aqueous sodium hydroxide (30 ml.), added in ten equal portions at 10 min. intervals. The product was then methylated three times with the Purdie reagents. The resulting syrup (0·187 g.) was refluxed with 4% methanolic hydrogen chloride (30 ml.) for 6 hr., the solution neutralised with silver carbonate, and the filtrate concentrated to a syrup. This syrup was heated with 2N-hydrochloric acid (20 ml.) for 2 hr. at 100°, the solution again neutralised, and the filtrate concentrated to a syrup. Fractionation of the methyl sugars on a cellulose column with butanol–ethanol–water (40 : 10 : 50 v/v) gave fraction I (41 mg.) and fraction II (14 mg.).

Fraction I was identical with 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose on paper chromatography and paper ionophoresis. On crystallisation, it had m. p. 90° undepressed on admixture with 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose and showed $[\alpha]_D^{18}$ +83·5° (equil.; *c* 1·00 in H_2O). Treatment with aniline gave crystals, m. p. 136° alone or in admixture with *N*-phenyl-2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucosylamine.

Fraction II moved slightly more slowly than 2 : 3-di-*O*-methyl-D-glucose on a paper chromatogram and had $[\alpha]_D^{18}$ +60·3° (equil.; *c* 0·60 in H_2O) (Found: OMe, 27·1. Calc. for a di-*O*-methylhexose: OMe, 29·8%). Fraction II was identical with authentic 3 : 6-di-*O*-methyl-D-glucose both on paper chromatography and ionophoresis and in its infrared absorption spectrum.

(viii) *Treatment with almond emulsin*. Trisaccharide A (20 mg.) was added to almond β-glycosidase solution (3 ml. in 0·5M-acetate buffer of pH 5·0). Components identical with glucose (trace), disaccharide X (trace), and trisaccharide A were detected after incubation for 3 days. The hydrolysis continued, until after 3 weeks' incubation *ca.* 50% of trisaccharide A remained. Under similar conditions cellobiose was completely hydrolysed in 3 days.

Characterisation of Disaccharide X.—(i) *Isolation*. Trisaccharide A (0·20 g.) was treated with almond β-glycosidase solution (12 ml. as above) and incubated at 37° for 6 weeks. The products (glucose, disaccharide X, and trisaccharide A) were separated [5, 6] on a charcoal–Celite column (*l*, 40 cm.; diam., 2 cm.) by gradient elution with aqueous ethanol (0 ⟶ 12%). The first fraction (0·08 g.) eluted contained glucose, $[\alpha]_D^{20}$ +52° (equil.; *c* 1·00 in H_2O), characterised as D-glucosazone, m. p. and mixed m. p. 202°. The second fraction (0·024 g.), eluted with 5—6·5% aqueous ethanol contained disaccharide X, had $[\alpha]_D^{20}$ +133° (equil.; *c* 0·94 in H_2O), and gave <0·5% of ash. The third fraction (0·092 g.), eluted with 10—12% aqueous ethanol, contained trisaccharide A and had $[\alpha]_D^{20}$ +92° (equil.; *c* 1·00 in H_2O).

(ii) *Characterisation*. Disaccharide X could be detected on paper chromatograms by

alkaline silver nitrate [17] and aniline hydrogen phthalate,[15] but not by alkaline triphenyltetrazolium chloride.[10] When oxidised by alkaline hypoiodite,[8] disaccharide X (3·4 mg.) consumed iodine equivalent to 1·76 mg. of glucose (98% of the theor. value for a disaccharide). When estimated by the cuprimetric Shaffer–Hartmann reagent,[9] disaccharide X (2·0 mg.) had a reducing power equivalent to 0·29 mg. of glucose (27·5% of the expected value for a disaccharide, a stoicheiometric reaction being assumed).

Complete hydrolysis of disaccharide X (as for trisaccharide A) gave glucose (identified on a paper chromatogram and ionophoretogram) equivalent to a 96% conversion. After oxidation of disaccharide X (4·2 mg.) with 0·075M-sodium periodate (10 ml.) for 15 hr., 6·1 moles of periodate were consumed and 2·85 moles of formic acid were produced per mole of disaccharide X. Disaccharide X alcohol (used above as a reference) was obtained by reduction of disaccharide X (4·0 mg.) with sodium borohydride (4·5 mg.) and isolated in a manner similar to that described above for trisaccharide A alcohol.

Oligosaccharide Synthesis in a Sucrose–Lactose Medium.—A sterile medium (250 ml.) was prepared containing yeast extract and salts as previously but with sucrose (2%) and lactose (10%), inoculated with *Betacoccus arabinosaceous* (Birmingham), and incubated at 25° for 3 days during which time the pH fell from 7·0 to 4·4. By a procedure similar to that used for trisaccharide A, a trisaccharide B (2·314 g.) was isolated from the culture fluid.

Characterisation of Trisaccharide B.—Unless otherwise stated the methods applied to trisaccharide B were the same as those for trisaccharide A.

(i) Trisaccharide B had $[\alpha]_D^{18}$ +103° (equil.; *c* 1·00 in H_2O) and gave <0·5% of ash. It moved as a single component when separated on a paper chromatogram (same solvent as above) either as the free sugar ($R_{glucose}$ 0·10) or as its benzylamine [14] derivative ($R_{glucose}$ 0·27). The free sugar was detectable by aniline hydrogen phthalate [15] but not by alkaline triphenyltetrazolium chloride.[10] On ionophoresis, the mobility of its borate complex was 0·33 and that of its *N*-benzylglycosylammonium ion [7] was 0·55.

(ii) *Oxidation by hypoiodite and copper reducing value.* With alkaline hypoiodite,[8] trisaccharide B (10 mg.) consumed iodine equivalent to 3·44 mg. of glucose (96% of the theor. value for a trisaccharide). When estimated by the Shaffer–Hartmann method,[9] trisaccharide B (20 mg. and 10 mg.) had a reducing power equivalent to 0·50 mg. and 0·24 mg. of glucose, respectively. This is 7·0% and 6·7% respectively of the calculated value for a trisaccharide.

(iii) *Hydrolysis.* Complete hydrolysis of trisaccharide B (1·86 mg.) gave a glucose–galactose mixture (identified on a paper chromatogram and ionophoretogram and estimated approx. as 2 : 1 ratio) which had a reducing power equivalent to 96% of the theoretical value. Paper chromatography of a partial hydrolysate of trisaccharide B showed components corresponding to glucose, galactose, lactose, trisaccharide B, and a disaccharide Y ($R_{glucose}$ 0·43). Ionophoresis in borate buffer (pH 10) showed components corresponding to glucose (M_G 1·00), galactose (M_G 0·93), lactose (M_G 0·38), and trisaccharide B (M_G 0·32). No separate spot was observed which could be attributed to disaccharide Y.

(iv) *Periodate oxidation.* Trisaccharide B (50·4 mg.), treated with sodium periodate as above, consumed 2·4 (5 min.), 3·05 (10 min.), 3·45 (20 min.), 3·6 (60 min.), 3·9 (300 min.), 3·9 (1080 min.) moles of periodate per mole. After 1080 min., 1·75 moles of formic acid were produced per mole. No formaldehyde was produced. Hydrolysis of the fully oxidised solution gave glucose.

(v) *Treatment with phenylhydrazine.* Osazones (0·37 g.) were formed from trisaccharide B (0·30 g.; method as above). After two recrystallisations from aqueous ethanol, the product (0·23 g.) was found to behave similarly to a mixture of lactosazone and glucosazone on ionophoresis in formate buffer (pH 1·8) and on circular paper chromatograms.

(vi) *Reduction of trisaccharide B.* Trisaccharide B (0·10 g.) was reduced with sodium borohydride to the alcohol (0·071 g.) which had $[\alpha]_D^{18}$ +99·5° (equil.; *c* 1·00 in H_2O) and moved as a single component ($R_{glucose}$ 0·16) in the solvent described above. It was not oxidised by alkaline hypoiodite [8] and had a negligible reducing power when determined by the Shaffer–Hartmann method.[9] Paper-chromatographic and -ionophoretic analyses of the complete hydrolysate of the alcohol indicated the presence of glucose, galactose, and glucitol. Similar analyses of the partial hydrolysate indicated the presence of glucose, galactose, glucitol, lactitol, disaccharide Y alcohol, and trisaccharide B alcohol.

[17] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.

Treatment of trisaccharide B alcohol (23·7 mg.) with sodium periodate resulted in the consumption of 3·65 (15 min.), 4·25 (60 min.), 4·58 (120 min.), 4·78 (180 min.), 4·90 (1200 min.) moles of periodate per mole. After 1200 min., 1·91 moles of formic acid and 0·85 mole of formaldehyde were produced per mole. Desalting and hydrolysis of the fully oxidised reaction solution yielded a component similar to a pentose on paper chromatography and identical with L-xylose on paper ionophoresis.

Incubation of trisaccharide B alcohol with almond β-glycosidase at 37° for 3 hr. gave a solution which, by paper chromatography, was found to contain galactose and disaccharide Y alcohol. Trisaccharide B alcohol was not detected in the digest after 72 hr.

Trisaccharide B alcohol (5·1 mg.) consumed 1·1 (15 min.), 1·1 (60 min.), 1·6 (180 min.), 2·7 (1080 min.), 4·9 (at 45° for 60 min.) moles of lead tetra-acetate per mole.

(vii) *Methylation.* Trisaccharide B (0·20 g.) was methylated as described above and the resulting syrup (0·17 g.) hydrolysed under the same conditions. Fractionation of the methyl sugars on a cellulose column gave fractions I (19·7 mg.), II (12·2 mg.), and III (15·8 mg.).

Fraction I was identical with 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose on paper chromatography and paper ionophoresis. On crystallisation, it had m. p. 88° undepressed on admixture with 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose and showed $[\alpha]_D^{18}$ +83° (equil.; *c* 0·80 in H_2O). The anilide (5 mg.) formed from fraction I (12 mg.) had m. p. 136° alone and in admixture with authentic *N*-phenyl-2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucosylamine.

Fraction II was identical with 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose on paper chromatography and ionophoresis. The anilide (3 mg.) formed from fraction II (8 mg.) had m. p. 190° alone and in admixture with authentic *N*-phenyl-2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactosylamine.

Fraction III had $[\alpha]_D^{18}$ +60° (equil.; *c* 0·50 in H_2O) and was identical with 3 : 6-di-*O*-methyl-D-glucose on paper chromatography and ionophoresis and in infrared absorption spectra.

(viii) *Treatment with almond emulsin.* Trisaccharide B (20 mg.) was added to almond β-glycosidase (3 ml.). After incubation at 37° for 7 days the presence of galactose, glucose (trace), disaccharide Y, and trisaccharide B was indicated by paper chromatography. The rate of hydrolysis was similar to that of trisaccharide A.

Characterisation of Disaccharide Y.—(i) *Isolation.* Trisaccharide B (0·50 g.) was treated with almond emulsin (0·50 g.) in 0·05M-acetate buffer of pH 5 at 37° for 4 weeks. After fractionation on a charcoal–Celite column (*l*, 40 cm.; diam. 2 cm.), the fraction eluted with 5—6% aqueous ethanol contained disaccharide Y (69·4 mg.).

(ii) *Examination.* On paper-chromatographic and -ionophoretic examination disaccharide Y moved in the same way as disaccharide X and an authentic specimen of 2-*O*-α-D-glucopyranosyl-D-glucose. None of the three was detectable by alkaline triphenyltetrazolium chloride [10] whereas maltose, nigerose, cellobiose, lactose, and isomaltose reacted readily with this reagent. Disaccharide Y had $[\alpha]_D^{20}$ +128° (equil.; *c* 1·00 in H_2O).

On oxidation with alkaline hypoiodite,[8] disaccharide Y (3·0 mg.) consumed iodine equivalent to 1·54 mg. of glucose (97·5% of the theoretical value). Examined by the Shaffer–Hartmann method,[9] disaccharide Y (1·80 mg.) had a reducing power equivalent to 0·22 mg. of glucose (23% of the expected value for a disaccharide). Analyses of the complete hydrolysate of disaccharide Y showed only glucose (96% conversion).

The infrared absorption spectra of disaccharide X, disaccharide Y, and 2-*O*-α-D-glucopyranosyl-D-glucose were identical.

Disaccharide Y (8·2 mg.) with 0·05M-sodium periodate (25 ml.) consumed 3·5 moles (15 min.) and 6·05 moles (22 hr.) of periodate per mole. After 22 hr., 3·1 moles of formic acid were produced per mole.

Disaccharide Y (15 mg.) was dissolved in water (1·5 ml.), and a solution of phenylhydrazine (0·3 ml.) in 50% acetic acid (1·5 ml.) was added. The solution was heated at 100° for 1 hr. and left overnight at 0°. The osazone (5·3 mg.), after two recrystallisations from ethanol, had m. p. 196—198° undepressed on admixture with D-glucosazone.

Reduction of disaccharide Y (15·2 mg.) with sodium borohydride (16 mg.) gave disaccharide Y alcohol (12·3 mg.) which had $[\alpha]_D^{20}$ +67° (equil.; *c* 0·60 in H_2O). Disaccharide Y alcohol (10·5 mg.) with 0·05M-sodium periodate (10 ml.) consumed 2·85 (15 min.), 4·15 (60 min.), 4·60 (180 min.), 4·90 (1080 min.) moles per mole. After 18 hr., 2·80 moles of formic acid and 0·90 mole of formaldehyde were produced per mole.

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