395. *Studies of* Aspergillus niger. *Part V.* The Enzymic Synthesis of a New Trisaccharide.*

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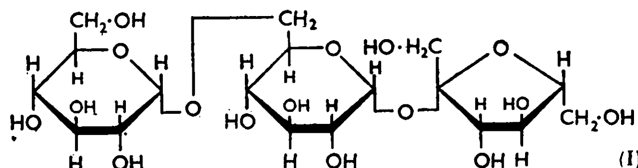
A trisaccharide produced by the action of *A. niger* " 152 " on a sucrose–maltose mixture has been characterised as *O*-α-D-glucopyranosyl-(1 ⟶ 6)-*O*-α-D-glucopyranosyl-(1 ⟶ 2) β-D-fructofuranoside.

In a search for possible precursors of nigeran, the polysaccharide synthesised [1] by *Aspergillus niger* " 152 ", the action of a cell-free extract of the mould on a mixture of sucrose and maltose was examined. From the complex mixture of oligosaccharides produced, we isolated a new trisaccharide (X), the structure of which we now report.

The mixture was separated by fractionation on a charcoal column [2] with successively smaller gradients of aqueous ethanol. The main trisaccharides produced were crystalline panose, a ketose that behaved on a paper chromatogram in the same way as *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside, and trisaccharide X. The first two presumably arose by transglucosidation from maltose and transfructosidation from sucrose, respectively.[3]

Trisaccharide X was rapidly hydrolysed by 0·1N-hydrochloric acid at 100° to *iso*maltose (isolated as β-*iso*maltose octa-acetate) and fructose. The observed optical rotation, $[\alpha]_D^{18}$ +46·7°, was in good agreement with that (+48·4°) calculated on the same basis for an equimolar mixture of *iso*maltose (+120°) and fructose (−92·4°) and the molar ratio aldobiose : ketose determined by Van der Plank's method [4] was 0·87 : 1. Since trisaccharide X was non-reducing and showed a low mobility on borate-buffer ionophoresis, this evidence and the conditions of its synthesis suggested that it was *O*-α-D-glucopyranosyl-



(1 ⟶ 6)-*O*-α-D-glucopyranosyl-(1 ⟶ 2) β-D-fructofuranoside (I). As would be expected, its optical rotation ($[\alpha]_D^{18}$ +102·5°) was slightly smaller than that of raffinose pentahydrate ($[\alpha]_D^{20}$ +105·2°) [cf. melibiose, +129·5° (equil.); *iso*maltose, +120° (equil.)]. Its infrared

* Part IV, *J.*, 1955, 3734.

[1] Barker, Bourne, and Stacey, *J.*, 1953, 3084.
[2] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[3] Barker and Carrington, *J.*, 1953, 3588; Barker, Bourne, and Carrington, *J.*, 1954, 2125.
[4] Van der Plank, *Biochem. J.*, 1936, **30**, 460.

spectrum was almost a summation of those of *iso*maltose and sucrose and showed a certain similarity to that of raffinose :

Trisaccharide X ...		919s			870m	852m	835m	803vw	769m
*iso*Maltose		919s					838m		768m
Sucrose	942m	919s	907s		865m	846m		797vw	
Raffinose	938s			888vw	872m	857m	830s		770m

Trisaccharide X should give, on periodate oxidation, the same hexa-aldehyde as raffinose [*O*-α-D-galactopyranosyl-(1 ⟶ 6)-*O*-α-D-glucopyranosyl-(1 ⟶ 2) β-D-fructofuranoside]. After treatment of trisaccharide X and of raffinose with periodate, the observed final optical rotations (+46·0°, + 48·2°), the formic acid produced (1·7, 1·9 mol.), and the periodate consumed (5·1, 5·2 mol.) were very similar.[5]

When the trisaccharide was methylated and then hydrolysed, paper chromatography showed the presence of 2 : 3 : 4-tri-*O*- and 2 : 3 : 4 : 6-tetra-*O*-methylglucose, a tetra-*O*-methylfructose, and traces of other products formed by incomplete methylation. This confirms the assigned structure.

The structure of trisaccharide X makes it probable that it was synthesised by the transglucosidase mentioned above, acting on maltose as substrate and using sucrose as receptor. Such a trisaccharide might be a possible intermediate in the synthesis of dextran from sucrose. White and Maher [6] isolated an analogue [*O*-α-D-glucopyranosyl-(1 ⟶ 4)-*O*-α-D-glucopyranosyl-(1 ⟶ 2) β-D-fructofuranoside] after treating sucrose with honey invertase.

EXPERIMENTAL

Enzymic Synthesis of Oligosaccharide Mixture (with T. R. CARRINGTON).—A freeze-dried cell-free extract [3] (5 g.) of *Aspergillus niger* " 152 ", dissolved in sterile distilled water (100 c.c.), was mixed with an aqueous sterile solution (300 c.c.) of maltose hydrate (50 g.) and sucrose (50 g.). After 70 hr. at 30° $[\alpha]_D^{17}$ had fallen to *ca.* +46·6° (*c* 5 in H_2O) and paper chromatography with the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) revealed components having R_F values identical with those of fructose, glucose, sucrose, maltose, *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside, panose, an unknown trisaccharide (X), and higher oligosaccharides. After adjustment of the pH to 7·2, enzyme action was arrested by 15 minutes' heating at 100°. After fractionation on a charcoal column [2] with gradient elution with aqueous ethanol,[7] the monosaccharides and most of the disaccharides (76·62 g.) were discarded. The trisaccharides were obtained in two fractions : A (10·69 g.), a mixture of maltose, *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside, trisaccharide X, and panose; and B (5·05 g.) which contained the same sugars together with higher oligosaccharides.

Isolation of Trisaccharide X.—Fraction A was refractionated on a charcoal column (65 × 4·6 cm. diam.) with gradient elution with 5—30% ethanol (8 l.), and freed from maltose and most of the *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside. The enriched fraction was freeze-dried to a powder (5·79 g.). The trisaccharides were extracted with boiling methanol (200 c.c.), and the solution set aside for 4 days at room temperature. Two crops (1·82 g.; 0·25 g.) of crystals (panose) were obtained, and the remaining trisaccharide mixture (3·68 g.) was refractionated on the charcoal column used above but with a lower gradient (5—15% ethanol; 8 l.). One fraction containing chromatographically pure trisaccharide X (0·225 g.) was obtained. The remaining fractions, when separated on a smaller charcoal column (39 × 2·5 cm. diam.) with a very small gradient (8·5—11% ethanol; 3 l.) afforded further pure trisaccharide X (0·476 g.) and a mixed fraction (0·273 g.) of panose and trisaccharide X.

Characterisation of Trisaccharide X.—(i) *Paper chromatography and ionophoresis.* In the solvent mixture described above, trisaccharide X moved as a single component (R_G 0·26) on a

[5] Cf. Hérissey, Wickstrom, and Courtois, *Bull. Soc. Chim. biol.*, 1951, **33**, 1768.
[6] White and Maher, *J. Amer. Chem. Soc.*, 1953, **75**, 1259.
[7] Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826.

paper chromatogram. The corresponding values for panose and *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside, were R_G 0·21 and R_G 0·34, respectively. The relative times needed for spots containing equal quantities of the three trisaccharides to develop their full intensity when sprayed with alkaline silver nitrate [8] were panose ⩽ *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside < trisaccharide X. On paper ionophoresis in borate buffer,[9] pH 10, the mobility of trisaccharide X was M_G 0·04 and that of panose M_G 0·19.

(ii) *Optical measurements.* Amorphous trisaccharide X showed $[\alpha]_D^{18}$ +102·5° (*c* 2 in H_2O). A small amount of trisaccharide X was crystallised from ethanol and had m. p. 118—120°. The infrared spectrum of the crystalline material in the region 1027—715 cm.$^{-1}$ showed absorption peaks at 978s, 919s, 870m 852m, 835m, 803vw, 769m cm.$^{-1}$.

(iii) *Partial acidic hydrolysis.* Trisaccharide X (57·8 mg.) was hydrolysed with 0·1N-hydrochloric acid (6 c.c.) at 100°. After 5 min. $[\alpha]_D^{18}$ had fallen from +102·5° to +46·7° and thereafter was constant for a further 5 min. The only components detectable by paper chromatography and ionophoresis were those having the same mobilities as fructose and *iso*maltose, together with traces of glucose and trisaccharide X. Quantitative determination [4] of the proportion of aldobiose to keto-sugar (approx. equal to the *iso*maltose : fructose ratio) in the partial hydrolysate after 7 min. gave a molar ratio 0·87 : 1. Further hydrolysis at 100°, after increase of the concentration of hydrochloric acid to N, hydrolysed the disaccharide, and only glucose and fructose were present in the final hydrolysate.

(iv) *Isolation of the products of partial hydrolysis.* Trisaccharide X (219 mg.) was hydrolysed with 0·1N-hydrochloric acid at 100° for 7 min. After neutralisation with silver carbonate, filtration, and concentration *in vacuo*, the products were fractionated on a charcoal–" Celite " column (40 × 2·5 cm. diam.). Washing with 1% aqueous ethanol (1 l.) eluted the monosaccharide fraction, which was concentrated to a syrup *in vacuo*, extracted with methanol, and de-ionised by treatment of the aqueous solution with small amounts of Amberlite IR-4B(OH^-) and Amberlite IR-120 (H^+). The freeze-dried product (68 mg., 87%) had $[\alpha]_D^{17}$ −84·9° (*c* 1 in H_2O) and behaved, on paper chromatography and paper ionophoresis, in the same way as fructose. Only traces of glucose were present.

The disaccharide fraction was eluted with 5% (v/v) aqueous ethanol (1 l.) and was purified as above. The freeze-dried product (121·2 mg., 82%) had $[\alpha]_D$ +119·2° (*c* 1·7 in H_2O) (Montgomery *et al.*[10] give +120° for *iso*maltose). In the region 1027—715 cm.$^{-1}$ its infrared spectrum was identical with that of *iso*maltose. Treatment of a portion (30 mg.) with acetic anhydride (1·5 c.c.) and sodium acetate (15 mg.) at 100—110° for 3 hr. gave crystalline β-*iso*maltose octa-acetate (5 mg.), m. p. and mixed m. p. 144—145°.

(v) *Periodate oxidation of trisaccharide X.* Trisaccharide X (40 mg.) and anhydrous raffinose (37·4 mg.) were severally dissolved in water (1 c.c.) and treated with 0·5M-sodium metaperiodate (1 c.c.) at 18°; changes in $[\alpha]_D^{18}$ were :

Time (min.)	0	3	7	20	60	120	300	600
Raffinose	+124·1°	+86	+78	+73	+64	+56	+50	+48·2
Trisaccharide X	+102·5°	+88	+76	+69	+62	+55	+47	+46·0

After 600 min., part (1·7 c.c.) of each solution was diluted to 5 c.c. Portions (2·5 c.c.) of these solutions were then used for determination of periodate consumption and formic acid (see above).

(vi) *Methylation of trisaccharide X.* The trisaccharide (25 mg.) was methylated in dioxan (2 c.c.) with ten portions each of 30% sodium hydroxide and dimethyl sulphate (total vol. 3·6 and 1·8 c.c. respectively) as described for kestose.[11] The product (10·5 mg.) was hydrolysed with 2N-sulphuric acid at 100° for 2 hr., then neutralised, and the filtrate freeze-dried. Paper chromatography, with naphtharesorcinol for selective identification of ketoses, showed components, *inter alia*, having R_F values of 2 : 3 : 4-tri- and 2 : 3 : 4 : 6-tetra-*O*-methylglucose and a tetra-*O*-methylfructose.

Characterisation of Panose.—The two crops of crystals isolated during separation of the oligosaccharide mixture were recrystallised from aqueous methanol and had m. p. 218—220°, undepressed on admixture with panose, and showed $[\alpha]_D^{18}$ +153·1° (equil.; *c* 1 in H_2O). On

[8] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[9] Foster, *J.*, 1953, 982.
[10] Montgomery, Weakley, and Hilbert, *J. Amer. Chem. Soc.*, 1949, **71**, 1682.
[11] Albon, Bell, Blanchard, Gross, and Rundell, *J.*, 1953, 24.

paper chromatography or paper ionophoresis its behaviour was identical with that of panose, and analysis of its partial hydrolysate showed the presence of *iso*maltose, maltose, glucose, and unchanged panose.

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