Studies of Aspergillus niger. *Part III.* The Structure of a Trisaccharide synthesised from Sucrose.*

By S. A. Barker, E. J. Bourne, and T. R. Carrington.

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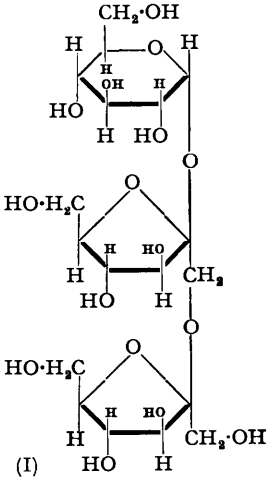
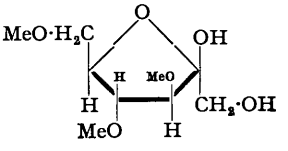
One of the trisaccharides produced from sucrose by *Aspergillus niger* (152) has been characterised as *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside.

During a preliminary investigation of the carbohydrate metabolism of *Aspergillus niger* (strain 152), which synthesises nigeran, a polyglucosan in which most, if not all, of the α-1 : 4- and α-1 : 3-linkages are arranged alternately (Barker, Bourne, and Stacey, *Chem. and Ind.*, 1952, 756; Part I, *J.*, 1953, 3084), two trisaccharides (termed I and II) were



(I)



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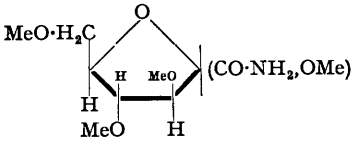


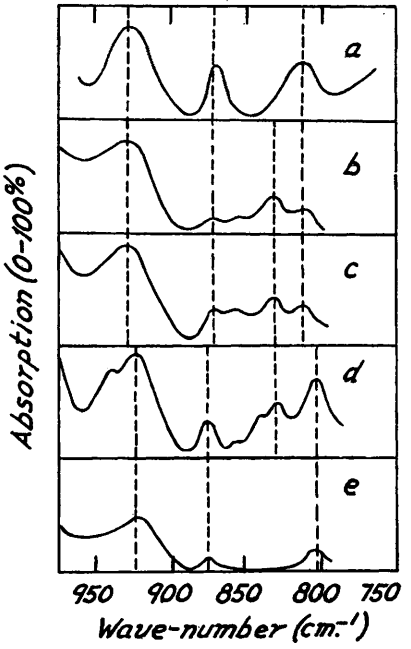
(III)

synthesised from sucrose by a cell-free extract of the mould (Barker and Carrington, Part II *). These trisaccharides, which were both non-reducing and contained two fructose residues and one of glucose, together with a tetrasaccharide fraction, containing

* Part II, *J.*, 1953, 3588.

three fructose residues and one of glucose, were considered to arise by a trans-fructosidase mechanism. The major component, trisaccharide I, has now been shown to be *O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(1 ⟶ 2) β-D-fructofuranoside (I).

The crystalline trisaccharide I was methylated, first with methyl sulphate–sodium hydroxide, and subsequently with methyl iodide–silver oxide, as described for the methylation of kestose [*O*-α-D-glucopyranosyl-(1 ⟶ 2)-*O*-β-D-fructofuranosyl-(6 ⟶ 2) β-D-fructofuranoside] by Albon, Bell, Blanchard, Gross, and Rundell (*J.*, 1953, 24). The resulting undeca-*O*-methyltrisaccharide, hydrolysed with 0·05N-sulphuric acid, gave sugars having R_F values and colour reactions with spraying reagents identical with those of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose, and 3 : 4 : 6-tri-*O*-methyl-D-fructose. Chromatography on a silica column (Bell and Palmer, *J.*, 1949,



a, *Inulin*.
b, *Trisaccharide I*.
c, *Trisaccharide* α_1.
d, *Kestose*.
e, *Levan*.

2522) was employed to separate the tri-*O*-methylfructose (0·95 mol.) from the two tetra-*O*-methyl sugars (1·85 mol.).

The tri-*O*-methyl sugar was characterised as 3 : 4 : 6-tri-*O*-methyl-D-fructofuranose (II) by its physical constants, by its ability to form an *iso*propylidene derivative, by its phenylosazone, and by oxidation with periodate. The sugar consumed 0·91 mol. of the oxidant, and gave formaldehyde (0·91 mol.), isolated as its crystalline dimedone derivative (cf. Bell, *J.*, 1948, 992), together with 2 : 3 : 5-tri-*O*-methyl-D-arabonolactone, characterised as the amide (cf. Hirst, Mitchell, Percival, and Percival, *J.*, 1953, 3170).

Determination of the aldose component of the mixture of tetra-*O*-methyl sugars with alkaline hypoiodite (cf. Hirst, McGilvray, and Percival, *J.*, 1950, 1297) showed the aldose and ketose sugars to be present in equimolecular proportions, a conclusion which was confirmed by the optical rotation of the mixture. Further fractionation was effected by mild treatment with methanolic hydrogen chloride and fractional distillation, to give tetra-*O*-methylglucose and methyl tetra-*O*-methylfructofuranoside (cf. Haworth and Mitchell, *J.*, 1923, 301). Crystallisation of the former gave 2 : 3 : 4 : 6-tetra-*O*-methyl-α-D-glucose, which was characterised also as its aniline derivative. The fructoside was hydrolysed to the free sugar, which was shown to be 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructofuranose by conversion into crystalline methyl 3 : 4 : 6-tri-*O*-methyl-D-fructofuronamide (III) (cf. Avery, Haworth, and Hirst, *J.*, 1927, 2308).

Partial hydrolysis of trisaccharide I gave fructose, glucose, and a non-reducing disaccharide, which, after purification on a charcoal column, could not be distinguished

from sucrose by ionophoresis or chromatography; it had $[\alpha]_D$ +69°, compared with +66·5° quoted for sucrose. Hydrolysis of the disaccharide caused inversion of the rotation, and gave fructose and glucose in approximately equimolecular proportions.

On the basis of the above observations, the trisaccharide was assigned the structure (I). The only dubiety which exists concerns the anomeric character of the linkage between the fructose units; however, it is most probable that this link is β in view of the prevalence of β-fructofuranoside residues in Nature. Such a structure is consistent with the view that the trisaccharide was formed by the transfer of a fructofuranose residue from one molecule of sucrose to another.

The synthesis of oligosaccharides from sucrose by extracts of moulds has been reported by several workers (cf., for example, Bealing and Bacon, *Biochem. J.*, 1951, **49**, lxxv; 1953, **53**, 277; Wallenfels and Bernt, *Angew. Chem.*, 1952, **64**, 28; Pazur, *J. Biol. Chem.*, 1952, **199**, 217), but only one product has yet been rigorously characterised (by Bacon and Bell, *J.*, 1953, 2528); this was a trisaccharide ("α_1") produced from sucrose by "Takadiastase" (a commercial mould enzyme preparation), and it was proved to have a structure identical with that now assigned to our own trisaccharide I. Further evidence that I and α_1 are the same trisaccharide is provided by their optical rotations (I, +29·2°; α_1, +30·5°, +32·6°), by those of their methyl ethers (I, +27·3°; α_1, +27·9°), by chromatography, and by their infra-red spectra (see Figure), determined over the frequency range 780—980 cm.$^{-1}$ by the Nujol "mull" technique (cf. Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171); the sample of α_1 was kindly provided by Dr. Bacon. It will be seen that these spectra show a closer correspondence with that of inulin, as regards both the positions and the relative intensities of the peaks, than with those of levan and kestose (kindly supplied by Dr. Gross), both of which have a β-2 : 6-fructosidic linkage. All three trisaccharide samples, and also sucrose, show an absorption peak at *ca.* 840 cm.$^{-1}$, whereas the two polysaccharides do not; this is a peak (type 2*a*) given by α-anomers in the glucopyranose series (cf. Barker *et al.*, *loc. cit.*, 1954).

Experimental

Isolation of the Trisaccharide.—Details for the preparation of the enzyme extract, its incubation with sucrose, and the detection and fractionation of the synthetic oligosaccharides were reported in Part II (*loc. cit.*). Trisaccharide I was crystallised from methanol–ethanol; it showed $[\alpha]_D^{16}$ +29·2° (*c*, 0·58 in H_2O), and was non-reducing to the Shaffer–Hartmann solution (*J. Biol. Chem.*, 1921, **45**, 377). Hydrolysis with 0·005N-sulphuric acid at 80° for 6 hr. (to $[\alpha]_D^{16}$ −36·2°) yielded a ketose and an aldose, in the molar ratio 2·1 : 1 (Van der Plank, *Biochem. J.*, 1936, **30**, 460), having R_F values identical with those of fructose and glucose, respectively, when irrigated on a paper chromatogram with the upper phase of *n*-butanol (40%)–ethanol (10%)–water (49%)–ammonia (1%). The ketose and aldose sugars were detected with naphtharesorcinol (Partridge, *ibid.*, 1948, **42**, 238) and aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443), respectively.

Methylation of the Trisaccharide.—In a typical experiment, the crystalline sugar (1·27 g.) was methylated in dioxan (20 ml.) at 30° with 30% sodium hydroxide solution (120 ml.) and methyl sulphate (60 ml.), as described for kestose (Albon *et al.*, *loc. cit.*). The product (0·945 g.) was thrice methylated under reflux with methyl iodide (4 ml.) and silver oxide (5·3 g.), and isolated from the silver residues by ether-extraction. The extract was filtered and evaporated at 30° *in vacuo* to a syrup (0·802 g., 48%), $[\alpha]_D^{20}$ +27·3° (*c*, 0·95 in $CHCl_3$), n_D^{28} 1·4590 (Found: C, 53·3; H, 8·3; OMe, 51·3. Calc. for $C_{29}H_{54}O_{16}$: C, 52·9; H, 8·3; OMe, 51·8%). Infra-red analysis showed negligible absorption in the region of the OH stretching frequency (3400—3700 cm.$^{-1}$), a critical test for free hydroxyl groups.

Hydrolysis of the Methylated Trisaccharide.—A solution of the methylated sugar (0·778 g.) in 0·05N-sulphuric acid (25 ml.) was heated at 95—100° for 75 min. (to $[\alpha]_D^{20}$ +43·9°, constant). After neutralisation with barium carbonate, paper-chromatographic analysis of the filtrate, as above, revealed a single aldose and two ketose components, identical in R_F values and spray reactions with reference spots of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose (R_F 0·86), 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose (R_F 0·88), and 3 : 4 : 6-tri-*O*-methyl-D-fructose (R_F 0·77). On the basis of the respective reported values for $[\alpha]_D$ [+81·3° (West and Holden, *Org. Synth.*, 1940, **20**, 97); +30·3° and +29·5° (Bell, *J.*, 1953, 1231)], the observed equilibrium value for an equimolar mixture of these sugars should have been +50·0°.

Chromatography on a column of silica gel (Bell and Palmer, *loc. cit.*) was employed to separate the tri-*O*-methylfructose from the tetra-*O*-methyl components. The silica gel (25 g.), prepared by Tristram's method (*Biochem. J.*, 1946, **40**, 723), was ground to a powder with water (15 ml.), suspended in toluene (100 ml.) containing 0·33% of ethanol, and closely packed into a column (12 × 3 cm.). The syrupy hydrolysate (0·773 g.) in toluene–ethanol (10 ml.) was allowed to diffuse into the column, which was developed with more toluene–ethanol (4 l.). The eluate was concentrated *in vacuo* at 30° over barium carbonate, filtered, and evaporated to a syrup (0·514 g.). Chromatographic analysis showed the presence of components corresponding to tetra-*O*-methyl-D-glucose and tetra-*O*-methyl-D-fructose, but none of the tri-*O*-methyl component. An aliquot of the syrup (9·53 mg.), treated with alkaline hypoiodite by a method essentially that of Hirst, McGilvray, and Percival (*loc. cit.*), was shown to contain 4·81 and 4·72 mg. (by difference) of the glucose and the fructose derivative, respectively. The mixture had $[\alpha]_D^{21}$ +54·5° (*c*, 0·95 in H_2O), compared with the theoretical value +55·8° for an equimolar mixture of tetra-*O*-methyl-D-glucose and tetra-*O*-methyl-D-fructose. The tri-*O*-methyl component was recovered from the column by elution with methanol (1·5 l.), and the eluate concentrated to a syrup (0·250 g.), shown to be chromatographically pure.

The mixture of tetra-*O*-methyl sugars was fractionated further by Haworth and Mitchell's method (*loc. cit.*). Accordingly, the syrup (0·386 g.) in dry methanolic hydrogen chloride (0·25% w/w; 20 ml.) was kept at room temperature until constant optical rotation (68·9°) was attained (5 days). The expected equilibrium rotation for the mixture, based on the $[\alpha]_D$ values of +81·3° (West and Holden, *loc. cit.*) and 57·7° (Haworth and Mitchell, *loc. cit.*), is +71·2°. The solution was neutralised with silver carbonate, filtered, and evaporated at 30° *in vacuo* to a syrup, which was fractionally distilled at 0·2 mm. The methyl tetra-*O*-methyl-D-fructofuranoside fraction (0·175 g.; b. p. 64—70°), collected in a receiver cooled in liquid air, was shown to be chromatographically pure (R_F 0·93); the residual tetra-*O*-methyl-D-glucose was contaminated with a little of the fructoside. A repetition of this experiment gave similar results.

*Characterisation of the Tri-*O*-methylfructose.*—(*a*) The syrup showed $[\alpha]_D^{20}$ +26·0° ⟶ +29·3°, 24 hr. (*c*, 0·62 in H_2O) (Found: OMe, 42·4. Calc. for $C_9H_{18}O_6$: OMe, 41·9%). Hirst, Mitchell, Percival, and Percival (*loc. cit.*) recorded $[\alpha]_D$ +27·0° ⟶ +29·0° (in H_2O) for 3 : 4 : 6-tri-*O*-methyl-D-fructose.

A portion (0·0377 g.) in anhydrous acetone (5 ml.) containing 0·5% of hydrogen chloride (w/w) showed $[\alpha]_D^{19}$ +31·8° ⟶ +63·7° (240 min.), indicating the formation of an *iso*propylidene derivative. Montgomery (*J. Amer. Chem. Soc.*, 1934, **56**, 419) gave $[\alpha]_D$ +70° for 3 : 4 : 6-tri-*O*-methyl-1 : 2-*O*-*iso*propylidene-D-fructofuranose.

(*b*) Oxidation of the tri-*O*-methylfructose (0·0245 g.) in 0·075M-phosphate buffer (10 ml.; pH 7·42) with 0·1M-sodium metaperiodate (5 ml.), by Bell's method (*J.*, 1948, 992), gave formaldehyde, isolated as the dimedone derivative (0·91 mol.), m. p. and mixed m. p. 189—191°. A further specimen (0·2544 g.), treated with 0·5M-sodium metaperiodate (15 ml.) in the dark at room temperature, by the method of Hirst *et al.* (*J.*, 1953, 3170), consumed 0·91 mol. of periodate (72 hr.), and gave 2 : 3 : 5-tri-*O*-methyl-D-arabonolactone (0·117 g.), which was characterised as the amide, m. p. 137—138°, not depressed in admixture with an authentic specimen (kindly provided by Mrs. E. E. Percival) (Found: C, 46·3; H, 8·1; N, 6·3. Calc. for $C_8H_{17}O_5N$: C, 46·4; H, 8·3; N, 6·8%).

(*c*) The sugar (0·076 g.) in water (3 ml.) and acetic acid (0·5 ml.) was heated at 70° for 2·5 hr. with phenylhydrazine (0·15 ml.), to give an osazone (0·046 g.), which after recrystallisation from anhydrous ether–light petroleum (b. p. 40—60°) had m. p. 130—132°, not depressed in admixture with an authentic specimen of 3 : 4 : 6-tri-*O*-methyl-D-fructose phenylosazone prepared by the late E. G. V. Percival.

*Characterisation of the Tetra-*O*-methylfructose.*—The methyl tetra-*O*-methyl-D-fructofuranoside (0·230 g.) was hydrolysed at 100° with 0·05N-H_2SO_4 (10 ml.), $[\alpha]_D^{18}$ +55·8° ⟶ +27·3° (0·5 hr.) (*c*, 4·60 in H_2O). Bell (*loc. cit.*) reported $[\alpha]_D$ +30·6° (equil., in H_2O) for 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose. The solution was neutralised with barium carbonate, filtered, and evaporated under diminished pressure, to give the tetramethyl ether as a syrup. A larger quantity was prepared in a second experiment. Oxidation of a sample (0·755 g.) with nitric acid (*d* 1·42; 8 ml.), followed by esterification, methylation, and fractional distillation (0·1 mm.), using the procedure of Avery, Haworth, and Hirst (*loc. cit.*), yielded two fractions (b. p. 75—82°, 0·282 g.; b. p. 82—90°, 0·302 g.). These were dissolved separately in methanol (3 ml.), saturated with ammonia (0°), and refrigerated (3 days). Evaporation of the solvent, and recrystallisation of the product from ethanol–ether, yielded, mainly from the second fraction,

methyl 3 : 4 : 6-tri-*O*-methyl-D-fructofuronamide (0·118 g.), m. p. 100—101°, alone and in admixture with an authentic specimen supplied by Mrs. E. E. Percival (Found : C, 48·2; H, 7·4; N, 5·7. Calc. for $C_{10}H_{19}O_6N$: C, 48·2; H, 7·7; N, 5·6%).

*Characterisation of the Tetra-*O*-methylglucose.*—(*a*) The tetra-*O*-methyl-D-glucose (0·431 g.), purified by distillation, was recrystallised from ether–light petroleum, and gave 2 : 3 : 4 : 6-tetra-*O*-methyl-α-D-glucose (0·195 g.), m. p. and mixed m. p. 86—89°, $[\alpha]_D^{19}$ +89·1° ⟶ +82·5° (24 hr.) (*c*, 0·61 in H_2O) (Found : C, 50·9; H, 8·6. Calc. for $C_{10}H_{20}O_6$: C, 50·8; H, 8·5%).

(*b*) The crystalline tetramethyl ether (0·110 g.), treated with aniline (Peat, Schlüchterer, and Stacey, *J.*, 1939, 581), gave 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-glucosylamine (0·019 g.), m. p. and mixed m. p. 133—135°.

Partial Hydrolysis of Trisaccharide I.—The trisaccharide (0·606 g.) was heated at 80° for 1 hr. with 0·005N-sulphuric acid (25 ml.). Only one disaccharide, having an R_F value and spray reactions identical with those of sucrose, was observed on a chromatogram of the neutral hydrolysate. This disaccharide was separated from the glucose and fructose also present by gradient elution (Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826) on a charcoal column, using 0—5% aqueous ethanol. Two fractions were obtained, one (0·425 g.) consisting of the monosaccharides, and the other (0·130 g.) containing the ionophoretically- and chromatographically-pure disaccharide. The disaccharide, hydrolysed with 0·05N-sulphuric acid at 90°, showed $[\alpha]_D$ +69·0° ⟶ −26·8° (2 hr.) (*c*, 0·52 based on reducing sugars in the hydrolysate). Analysis of the hydrolysate (see above) showed the molar ratio of ketose to aldose to be 1·18 : 1; these two sugars were chromatographically identical with fructose and glucose, respectively.

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CHEMISTRY DEPARTMENT, THE UNIVERSITY,
EDGBASTON, BIRMINGHAM 15.

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