4. *Kestose : a Trisaccharide formed from Sucrose by Yeast Invertase.*

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The trisaccharide kestose, one of the oligosaccharides synthesised during the action of yeast invertase preparations on concentrated sucrose solutions, has been isolated by chromatography on a cellulose column. It is composed of two radicals of D-fructose and one of D-glucose; some of its properties have been determined. Analysis of the cleavage products of the methylated sugar show that it is *O*-α-D-glucopyranosyl(1→2)*O*-β-D-fructofuranosyl(6→2)β-D-fructofuranoside.

As a result of specificity (cf. Levi and Purves, *Adv. Carbohydrate Chem.*, 1949, **4**, 1; Gottschalk, *ibid.*, 1950, **5**, 49) the invertase of yeast is considered to be a β-fructofuranosidase, *i.e.*, it specifically attacks the fructofuranoside linkage in the sucrose molecule. The progress of sucrose hydrolysis has usually been followed polarimetrically in the assumption that equal parts of glucose and fructose are formed. However, Bacon and Edelman (*Arch. Biochem.*, 1950, **28**, 467) and Blanchard and Albon (*ibid.*, 1950, **29**, 220) showed that incomplete hydrolysis of sucrose by a variety of yeast invertase preparations produced, in addition to fructose and glucose, a number of higher oligosaccharides which were themselves eventually broken down to fructose and glucose. These results were confirmed by Fischer, Kohtès, and Fellig (*Helv. Chim. Acta*, 1951, **34**, 1132) using a highly purified yeast enzyme preparation. White and Secor (*Arch. Biochem. Biophys.*, 1952, **36**, 490) have determined the ratios of fructose to glucose in a number of these oligosaccharides. Several authors have suggested that invertases should be regarded as fructose radical-transferring enzymes, and Bacon (*Biochem. J.*, 1952, **50**, xviii) has demonstrated that a yeast invertase preparation can transfer these radicals from sucrose to various alcohols to form fructosides. Barton-Wright and Harris (*Nature*, 1951, **167**, 560) have shown that oligosaccharide excretion takes place when yeasts are grown on a sucrose medium.

A similar fructose-transferring effect has also been observed during the action of mould preparations on sucrose (Bealing and Bacon, *Biochem. J.*, 1951, **49**, lxxv; Pazur, *Fed. Proc.*, 1952, **11**, 267). Again the oligosaccharides were themselves eventually completely hydrolysed. Bealing and Bacon (*Biochem. J.*, 1952, in the press) have concluded that the

mould invertase responsible for this effect is a β-fructofuranosidase similar to, but not identical with, that of yeast invertase.

Preliminary work showed that oligosaccharides were produced in a yield of at least 6% (calculated on the original sucrose) when yeast invertase acted on concentrated sucrose solutions, the greatest amount being present when about 50% of the sucrose had been consumed ("50% hydrolysis"). When no sucrose remained, no oligosaccharides were detectable. The method of preparation of the enzyme did not appear to matter; a number of different yeast preparations gave identical results.

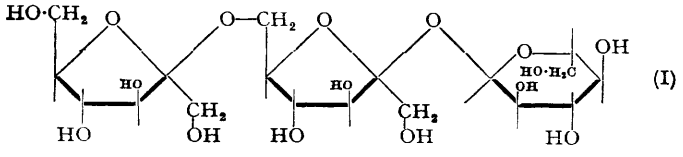
In the present preparative work, a 50% (w/w) sucrose solution was "50% hydrolysed" by a commercial yeast invertase preparation at pH 4·5 and the product analysed by paper-chromatography (de Whalley, Albon, and Gross, *Analyst*, 1951, **76**, 287; Albon and Gross, *ibid.*, 1952, **77**, 406, 410). This led to three oligosaccharide spots in addition to those of sucrose, fructose, and glucose. Using Bacon and Edelman's nomenclature (*loc. cit.*) we have termed these spots components III, II, and I; their respective R_F^{22} values were 0·12, 0·14, and 0·19, that of sucrose being 0·24. The product from 60 g. of sucrose was then separated on a large column of powdered cellulose (Hough, Jones, and Wadman, *Nature*, 1948, **162**, 448; *J.*, 1949, 2511) as modified for large-scale separation by Gross and Albon (*Analyst*, 1952, in the press). Component III was obtained from a number of fractions. Crystallised from methanol it was chromatographically at least 95% pure and gave a positive Raybin reaction (Raybin, *J. Amer. Chem. Soc.*, 1933, **55**, 2603; 1937, **59**, 1402). Hydrolysis to completion by yeast invertase produced two parts of fructose and one of glucose, but paper chromatograms at intermediate stages revealed spots in the position expected for sucrose. A molecular-weight determination confirmed the trisaccharide structure.

A report on the industrial significance of this sugar, to which the name kestose has been assigned, has already appeared (de Whalley, *Int. Sugar J.*, 1952, **54**, 127).

Kestose was methylated in the usual way, in good yield, to an undecamethyl ether, which was hydrolysed by 0·04N-sulphuric acid to 1 : 3 : 4 : 6-tetramethyl fructose, 2 : 3 : 4 : 6-tetramethyl glucose, and 1 : 3 : 4-trimethyl fructose (detected chromatographically on paper). The rotation of the hydrolysate indicated that the three components were present in equimolecular amounts. Quantitative chromatography (Bell and Palmer, *J.*, 1949, 2522) separated the trimethyl component as pure crystalline 1 : 3 : 4-trimethyl-D-fructose, and, judged from its specific rotation, the residue was an equimolar mixture of the tetramethyl components.

An attempt was made to separate the tetramethyl sugars (280 mg. in all) by Haworth and Mitchell's procedure (*J.*, 1923, **123**, 308). Control experiments showed that the 1 : 3 : 4 : 6-tetramethyl methylfructofuranoside formed was so volatile as largely to escape condensation even at low temperatures and moderately reduced pressures. The tetramethyl glucopyranose was, however, recovered crystalline. The methylated kestose products yielded crystalline 2 : 3 : 4 : 6-tetramethyl D-glucopyranose, but the ketoside recovered was insufficient for certain identification. The polarimetric behaviour of the mixed sugars in dry methanol containing 0·25% of hydrogen chloride was, however, fully consistent with that of equimolar proportions of the two sugars named above.

We therefore consider that kestose originates in the catalytic addition to sucrose of a fructofuranose radical transferred from a second sucrose molecule (cf. White, *Arch.*



(I)

Biochem. Biophys., 1952, **39**, 238). From the known specificity of yeast invertase, the newly formed fructoside link must be presumed to be of the β-type. Kestose would therefore be *O*-α-D-glucopyranosyl(1→2)*O*-β-D-fructofuranosyl(6→2)β-D-fructofuranoside (I)

A similar trisaccharide ($[\alpha]_D^{20}$ +23°) has been isolated by Dedonder (*Compt. rend.*,

1951, **232**, 1134; *Bull. Soc. Chim. biol.*, 1952, **34**, 144) from an extract of tubers of the Jerusalem artichoke, and termed by him "Glucofructosane B." It seems probable (cf. also Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114) that the carbohydrates of such tubers form a series structurally related to inulin, and it might be expected that the trisaccharide found by Dedonder would possess the typical 2 : 1′-link between the fructose radicals. However, Pazur (personal communication) has isolated from the products of the action of a mould extract on sucrose a trisaccharide ($[\alpha]_D$ +17°) to which he ascribes the 2 : 1′-linkage. This may be identical with component II produced by the yeast invertase reaction, which is present in rather smaller amount than is kestose.

Experimental

Evaporations were conducted below 45° with adequate pH control. Rotations were determined in 2-dm. tubes. Elementary analyses were by Drs. Weiler and Strauss, Oxford.

Paper-chromatographic Examination of Partly Inverted Sucrose.—The solvent was *n*-propanol–ethyl acetate–water (7 : 2 : 1, by vol.). Chromatograms were run for 48 hours at 22° and sprayed (for ketoses) with a 10 : 1 mixture of 2% alcoholic α-naphthol and phosphoric acid. For R_F values see p. 25.

Large-scale Fractionation of Partly Inverted Sucrose.—A solution of sucrose (60 g.) in water (54 ml.) was adjusted to pH 4·5 with acetic acid. "Sumasuco" Invertase Concentrate (Sugar Manufacturers Supply Co.) (6 ml.) was added and the solution kept at 20° for 40 minutes (approx. time for 50% inversion). The reaction was then stopped by boiling for a few seconds. A glass tube of 5-in. diameter was packed to a depth of 12·5 in. with ashless cellulose powder (Whatman standard grade) (*ca.* 1·7 kg.), and the column washed with de-aerated water followed by the eluting solvent (*iso*propanol–butanol–water, 7 : 1 : 2 by vol.). The partly inverted sucrose, dissolved in this solvent (800 ml.), was placed on the column, allowed to soak in, and eluted at the rate of 100 ml. per hour. Fractions of 80 ml. (14 l. in all) were collected by means of an automatic drop-counting fraction-collector. Elution of the sugars was followed by paper chromatography. The fractions from 9—12 l. of the eluate contained chromatographically pure kestose (component III).

Isolation of Kestose.—The fractions containing kestose alone were evaporated, taken up in methanol (70 ml.), and again evaporated to remove water and butanol. A solution of the residue in methanol (140 ml.) was filtered, concentrated to 20 ml., and kept in a desiccator (silica gel) at room temperature; the *kestose* (1·75 g.) which then crystallised was washed with methanol and dried to constant weight *in vacuo* at 50°, showing no loss in weight at 105°. It had m. p. 145° ± 2° (uncorr.), $[\alpha]_D^{20}$ +27·3° (*c*, 2·19 in H_2O) (Found: C, 42·3; H, 6·5. $C_{18}H_{32}O_{16}$ requires C, 42·9; H, 6·4%). The molecular weight (489; error ±4%) ($C_{18}H_{32}O_{16}$ requires 504) was determined by an unpublished cryoscopic semimicro-method due to Gross, a thermistor resistance thermometer (Standard Telephones and Cables Ltd.) being used after calibration against a Beckmann thermometer.

The sugar was very soluble in water and was not sweet. It crystallised from methanol and (with some difficulty) from water as fragile rhomboidal plates. It was non-reducing to Fehling's solution and ammoniacal silver nitrate under mild conditions. It gave a positive Raybin reaction. After complete hydrolysis by yeast invertase the specific rotation of the reaction mixture agreed with a mixture of two parts of fructose and one of glucose. This was confirmed by chromatographic comparison, and by estimating (*a*) total reducing power (Somogyi, *J. Biol. Chem.*, 1937, **117**, 771) and (*b*) ketose, as fructose (Roe, *J. Biol. Chem.*, 1934, **107**, 15).

Methylation.—All organic solvents used were distilled from glass apparatus. To kestose (1 g.), in dioxan (10 ml.) and 30% (w/v) sodium hydroxide solution (10 ml.), rapidly stirred at room temperature, were added, at 10-min. intervals, 30% sodium hydroxide solution (80 ml.) and methyl sulphate (40 ml.), each in 10 equal portions. After 0·5 hour's heating of the stirred reaction mixture at ~100°, the product was isolated by three partitions into chloroform. After evaporation of the solvent, the crude methylated kestose was thrice treated with Purdie's reagents, the final product (0·77 g.; average loss per treatment ~10%) being isolated from a ethereal solution (charcoal) and dried (P_2O_5–NaOH) in a high vacuum. The resulting *undecamethyl kestose* was a non-reducing syrup, $[\alpha]_D^{18}$ +25·8° (*c*, 4·2 in H_2O), +20·1° (*c*, 3·5 in $CHCl_3$), n_D^{20} 1·4060 (difference per 1°, ±0·00036) (Found: C, 52·0; H, 8·3; OMe, 51·8. $C_{29}H_{54}O_{16}$ requires C, 52·3; H, 8·2; OMe, 51·8%).

Hydrolysis of Methylated Kestose and Paper Chromatography of the Hydrolysate.—The ether (0·693 g.) in 0·04N-sulphuric acid (25 ml.) was heated at ~100° until a constant optical rotation

was observed (60 min.; $[\alpha]_D^{20}$ +19° *c*, 4). A specimen of the evaporated solution, neutralised by barium carbonate and filtered, was examined by paper chromatography. A single aldose, corresponding to 2 : 3 : 4 : 6-tetramethyl glucose, and two ketoses corresponding to 1 : 3 : 4 : 6-tetramethyl fructose and 1 : 3 : 4-trimethyl fructose were the sole components of the mixture. These three sugars (in the D-configuration) in equimolar proportions would show $[\alpha]_D^{20}$ +18·7° (in H_2O), a figure calculated from the following respective values found at 20·0° for chromatographically pure materials: +81·5° (West and Holden, *Org. Synth.*, 1940, **20**, 97; Greville and Northcote, *J.*, 1952, 1945); −55·7° (Bell, unpublished); +30·3° (Bell, unpublished). Tetramethyl derivatives of D-glucofuranose and of D-fructopyranose could not have been present in detectable amounts. Aniline phthalate and resorcinol–hydrochloric acid sprays were used to detect aldoses and ketoses respectively (cf. Bell and Palmer, *loc. cit.*).

Identification of the Cleavage Products of Methylated Kestose.—15 Ml. of the above hydrolysate were evaporated and the resulting syrup was chromatographed (Bell and Palmer, *loc. cit.*) on a 15-g. silica–water column, the mobile phase being 30 column-lengths of toluene–0·33% ethanol. Evaporation of the eluate yielded tetramethyl hexoses (280 mg.), $[\alpha]_D^{20}$ 55·0° in H_2O, corresponding to an equimolar mixture of 2 : 3 : 4 : 6-tetramethyl D-glucose and 1 : 3 : 4 : 6-tetramethyl D-fructose. Paper chromatograms showed two spots only, which corresponded exactly to these two substances. The aqueous solution of this mixture, which had been used to determine the specific rotation, was evaporated by freeze-drying; even then some of the fructose component volatilised (Bell and Palmer, *loc. cit.*). The dried material was dissolved in dry methanol (25 ml.) containing 0·25% of hydrogen chloride (w/w) and the polarimetric behaviour observed in a 4-dm. tube at 18°. The initial $[\alpha]_D$ (+52·5°) (calc. from approx. wt. of sugars) rose to a constant value of +70° in 24 hours. This behaviour is expected from an equimolar mixture of the suspected two sugars (cf. Haworth and Mitchell, *loc. cit.*). After isolation in the usual way, the reaction product was heated at 70°/0·1 mm. under a condenser cooled by acetone–solid carbon dioxide. The non-volatile residue crystallised; after trituration with a little light petroleum (b. p. 40—60°) the crystals melted at 94—96° alone or mixed with authentic 2 : 3 : 4 : 6-tetramethyl D-glucose. The amount of the distillate was too small to permit more than paper chromatographic examination, after appropriate hydrolysis; a single spot identical with 1 : 3 : 4 : 6-tetramethyl fructose was obtained. A control experiment with the same amount of an equimolar mixture of the two tetramethyl sugars gave similar results.

A methanolic extract of the stationary phase of the silica column yielded a colourless syrup (130 mg., *ca.* 100%) which rapidly crystallised. Paper chromatography indicated that 1 : 3 : 4-trimethyl fructose was the sole component. The material, without further recrystallisation, had n_D^{20} 1·4658, m. p. 68—71° alone or mixed with 1 : 3 : 4-trimethyl D-fructose (m. p. 70—73°), $[\alpha]_D^{19}$ −55·0° (*c*, 2·5 in H_2O; equil.). Chromatographically pure 1 : 3 : 4-trimethyl D-fructose, examined under identical conditions, had $[\alpha]_D^{19}$ −55·2°.

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