neo*Kestose: A Trisaccharide Formed from Sucrose by Yeast Invertase.*

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[Reprint Order No. 5032.]

A non-reducing trisaccharide, synthesised during the action of yeast invertase preparations on sucrose, has been isolated by chromatography on a carbon–Celite column together with ~10% of the known isomeric trisaccharide, Gl α—β2-Fru*f*1—β2-Fru*f*. The new substance is composed of two D-fructose and one D-glucose radicals. From analysis of the cleavage products of the mixed methylated sugars the following structure has been assigned to the new trisaccharide; *O*-β-D-fructofuranosyl(2→6)-α-D-glucopyranosyl(1→2) β-D-fructofuranoside. It appears to be formed by enzymic transfer of a β-D-fructofuranosyl radical to sucrose. The common name *neo*kestose is suggested. Confirmation is thus provided that invertase will transfer β-D-fructofuranosyl radicals to the primary alcoholic group of D-glucopyranose as well as to those of D-fructofuranose.

A PREVIOUS communication (Albon, Bell, Blanchard, Gross, and Rundell, *J.*, 1953, 24) reported the isolation and characterisation of one of the several trisaccharides synthesised by yeast invertase preparations acting on sucrose. In Bacon and Edelman's nomenclature (*Arch. Biochem.*, 1950, **28**, 467) this was " Component III " and was named kestose (II) (de Whalley, *Int. Sugar J.*, 1952, **54**, 127). We have since tried to isolate a sufficient amount of another non-reducing trisaccharide, " Component II," which according to preliminary work was known to be formed along with kestose and to consist likewise of two fructose and one glucose radical (White and Secor, *Archiv Biochem. Biophys.*, 1952, **36**, 490). Component II was present in the incomplete enzymic hydrolysate of sucrose at a concentration far lower than kestose; since the R_F values of Components III and II are close, the separation of sufficient material for structural analysis and determination of some physical constants did not appear to be simple. On a large cellulose column (Albon *et al.*, *loc. cit.*) only a small proportion of Component II was recovered in (apparently) homogeneous fractions, whilst the bulk of it was found in fractions mixed mainly with kestose, " Component I," and sucrose.

Preliminary small-scale attempts to separate Component II on charcoal–Celite columns (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677) proved more successful and revealed an interesting reversal of the sequence of eluted components in that Component II appeared in the eluate after Component III (kestose).

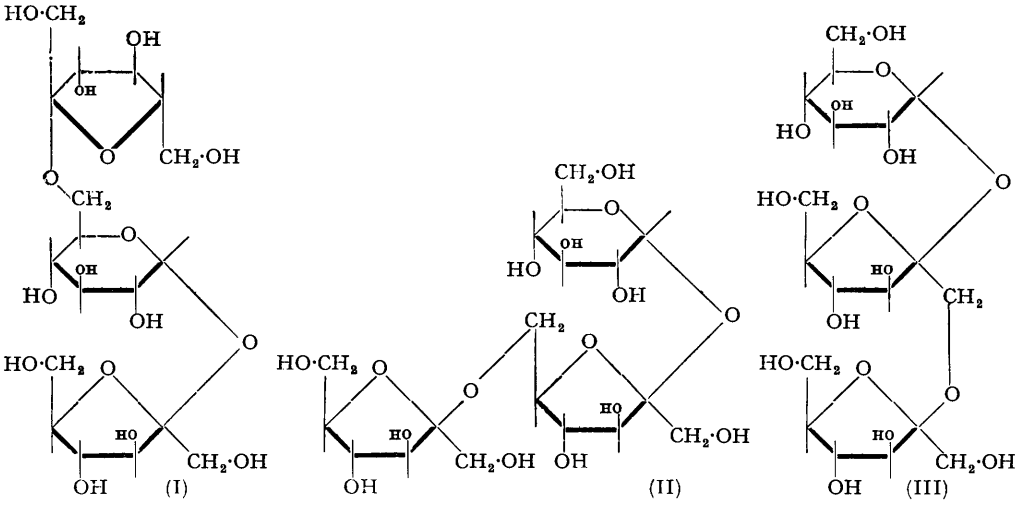
It was thought that this effect itself combined with the recently described " gradient elution " technique (Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826; Bacon and Bell, *J.*, 1953, 2528) might facilitate a satisfactory separation. A 50% solution (w/w) sucrose solution, " 50% hydrolysed " by a yeast invertase preparation at pH 4·6, subjected to chromatographic fractionation by this technique, yielded Component II in a " chromatographically pure state," *i.e.*, paper-chromatographic examination of the material recovered showed it to be identical with Component II and to give a single spot.

The product could not be crystallised from water, methanol, or ethanol; it was obtained as a white, non-reducing, amorphous powder by grinding the dried eluate with dry acetone; after drying to constant weight a very hygroscopic material was obtained, having $[\alpha]_D^{20}$ +22·2° (in H_2O). Hydrolysis to completion by yeast invertase yielded 2 mols. of fructose and 1 mol. of glucose per mol. of trisaccharide.

Methylation of " Component II," as for kestose (Albon *et al.*, *loc. cit.*) and for its isomeric fructosylsucrose (Bacon and Bell, *loc. cit.*), yielded a product whose analysis suggested a hendeca-*O*-methyltrisaccharide. When the acid hydrolysate (0·05N-sulphuric acid) was analysed on a silica partition column (Bell and Palmer, *J.*, 1949, 2522; Albon *et al.*, *loc. cit.*) two clear-cut fractions were obtained. The first was expected to contain only 1 : 3 : 4 : 6-tetra-*O*-methylfructofuranose and the second only 2 : 3 : 4-tri-*O*-methyl-D-glucose. How-

ever, the physical constants of both fractions indicated that neither was homogeneous (cf. Bell and Palmer, *J.*, 1952, 3763) and paper chromatography confirmed this. The first fraction was assayed as containing ~90% of 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose contaminated by ~10% of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. The second fraction was assayed as containing ~90% of 2 : 3 : 4-tri-*O*-methyl-D-glucose and ~10% of 3 : 4 : 6-tri-*O*-methyl-D-fructose. It therefore was clear that " Component II," as isolated above, was not a homogeneous trisaccharide.

However, examination of Component II by high-voltage paper electrophoresis of carbohydrates in borate buffers (Gross, *Nature*, 1953, **172**, 908) revealed the presence of a minor



" spot " as well as a major " spot "; the latter must have represented the bulk of the material. While the mobility of the minor spot was slightly lower than that of kestose, that of the major spot was considerably lower, thus affording an easy and complete separation of it from either of the two former compounds (Gross, *Nature*, 1954, **173**, 487). While this work was in progress Dr. J. S. D. Bacon informed us that separation of Component II into 2 constituents, II_1 and II_2, had been achieved by the gradient-elution technique on a carbon–Celite column. A sample of II_2 (received from Dr. J. S. D. Bacon) showed the same electrophoretic behaviour as the major spot and a sample of II_1 corresponded to the minor spot. By this time it was too late to attempt to separate Component II on the basis of the new information, since the bulk of it had already been subjected to methylation. On the other hand our preparation and that of Bacon need not have been identical in their proportions of II_2 and II_1 since the conditions for incubation of the original solutions were different and might have caused the presence of a larger amount of II_1 in Bacon's case. The concentration of II_1 (~5—8%) in our product was estimated by visual comparison with a series of standards, but contamination, regrettable though it was, did not however seriously affect the determination of the structure of the main component, the so-called " II_2." The results of the methylation–hydrolysis–chromatographic procedure thus confirmed the findings of paper electrophoresis of the sugar–borate complexes.

It can be stated with certainty, after indubitable characterisation of the major components of each fraction, that approximately 90% of our " Component II " formed by the action of yeast-invertase preparations on sucrose consists of a new trisaccharide having the structure *O*-β-D-fructofuranosyl(2→6)-α-D-glycopyranosyl(1→2) β-D-fructofuranoside (I), and that the 10% impurity consists of *O*-α-D-glucopyranosyl(1→2)-β-D-fructofuranosyl-(1→2) β-D-fructofuranoside (III) (Bacon and Bell, *loc. cit.*, Bacon, *Biochem. J.*, in the press). The β-fructofuranosyl linkages would almost certainly follow from the known specificity of the invertases. It therefore seems clear that this experiment has confirmed the hypothesis

of Edelman and Bacon (*Biochem. J.*, 1951, **49**, 529), Fischer *et al.* (*Helv. Chim. Acta*, 1951, **34**, 1132), Bacon (*Biochem. J.*, 1952, **50**, xviii), and Whelan and Jones (*Biochem. J.*, 1953, **54**, xxxiv) that invertases (whatever their origin) have a property in common, namely, the ability to transfer β-D-fructofuranosyl radicals to acceptors which are (*a*) water molecules or (*b*) molecules of primary alcohols.

The nomenclature of new compounds always presents a problem. The first of the present series, namely, Gl α-β-2 Fru*f* 6—β2-Fru*f* (II), has been called kestose. Gl α—β 2-Fru*f* 1—β2-Fru*f* (III) is so-far unnamed. (I) cannot logically be called *iso*kestose since the name *iso*sucrose has been adopted for an isomer having linkages anomerically different from sucrose. The name kestose, having thus become established, presents a difficulty. It is therefore suggested that the substance (II) hitherto known as kestose be known as 6-kestose, the substance characterised by Bacon and Bell (*loc. cit.*) (III) as 1-kestose, and the substance (I) which is the subject of the present communication as *neo*kestose.

Experimental

Specific rotations were determined in water in a 2-dm. tube unless otherwise stated. Evaporations were done below 45° with adequate pH control.

The following values for $[\alpha]_D^{20}$ and n_D^{20} were used as standards: (*a*) 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose: $[\alpha]_D^{20}$ +81·5° (Greville and Northcote, *J.*, 1952, 1945), n_D^{20} 1·4555 (present work); (*b*) 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose: $[\alpha]_D^{20}$ +30·3°, n_D^{20} 1·4506 (Bell, *J.*, 1953, 1231); (*c*) 2 : 3 : 4-tri-*O*-methyl-D-glucose: $[\alpha]_D^{20}$ +78·7°, n_D^{20} 1·4700 (Greville and Northcote, *loc. cit.*); (*d*) 3 : 4 : 6-tri-*O*-methyl-D-fructose: $[\alpha]_D^{20}$ +29·5°, n_D^{20} 1·4651 (Bell, *loc. cit.*).

Preparation of Crude Component II.—An undialysed commercial invertase preparation ["Sumasuco" Invertase Concentrate (Sugar Manufacturers Supply Co., London)] (2·5 ml.) was allowed to act at 20° for 45 min. on a solution of sucrose (27·5 g.) in water (22·5 ml.) and 0·2M-acetate buffer (2·5 ml.) of pH 4·6. These conditions were chosen to give "50% inversion." The enzyme was then inactivated by boiling and the solution stored in an ice-box.

Large-scale Fractionation.—For fractionation by "gradient elution," three active carbons were tried; "activated charcoal" (British Drug Houses Ltd.) "active carbon No. 130" (Sutcliffe, Speakman & Co. Ltd.), and "Darco G 60" (Atlas Powder Co., New York). Active carbon No. 130 was finally chosen, as it gave a neutral eluate (as did also Darco G 60) and was not noticeably inferior to the others in resolving power. A glass tube was packed with a carefully mixed aqueous slurry of 160 g. of active carbon No. 130 and 160 g. of Celite No. 535 (Johns Manville Co. Ltd.) to form a column 70 mm. in diam. and 225 mm. high. It was first washed with water (pH of eluate 6·8), then with 30% industrial alcohol, and again with water. After reaction the sucrose solution (27 ml.) was placed on top of the column, allowed to soak in, and eluted with water of steadily increasing alcohol content. This was done by connecting the liquid on top of the column through a narrow syphoning tube with a closed 2-l. flask containing water and fitted with an electrically driven stirrer running in Polythene bearings. This flask was connected through a syphoning tube with another 2 l. flask containing 47·5% aqueous ethanol (v/v). This concentration was calculated to give a gradient reaching 30% alcohol content after 2 l. of solvent had left the first flask.

The flow rate was maintained at 60 ml./hr. and the average volume of the automatically collected fractions was 15 ml. The resolving power of the column was first tested with a mixture containing 5·5 g. of sucrose (after reaction), an amount well below the expected "loading capacity" of the column. Monosaccharides were eluted by the first 1380 ml. Mixed fractions containing disaccharides emerged up to 1770 ml., then fractions containing a mixture of disaccharides (mainly sucrose) and kestose, up to 1900 ml. After a very small gap, Component II emerged in chromatographically "homogeneous" fractions up to 2500 ml.

As the aim of this work was to obtain the maximum quantity of "Component II," the column was thereafter overloaded with 27·5 g. of "reacted sucrose." A consequent decrease in resolving efficiency necessitated the discarding of 10 overlapping fractions which contained mainly sucrose with some kestose and Component II.

After evaporation of the bulked "homogeneous" fractions to a thick syrup and their redissolution in water, a solid residue was left. This appeared to be silica originating from the column. Only after repeated evaporation and extraction with water could this contaminant be eliminated. The composition of the carbohydrate finally obtained was checked by elementary analysis.

The yields of Component II from five runs were combined and evaporated to a thick syrup. This was dissolved in water (5 ml.) and rechromatographed on a carbon–Celite column (described above). Paper chromatography of highly concentrated samples of the rechromatographed Component II revealed the presence of small amounts of sucrose and a very small amount of a substance appearing in the position of Component IV. To eliminate these contaminants, the material (1·8 g.) was dissolved in *iso*propanol–butanol–water (7 : 1 : 2 by vol.) (65 ml.) and passed through a powdered celluose column, 45 mm. in diam. and 250 mm. high (Gross and Albon, *Analyst*, 1953, **78**, 191), fractions of 15 ml. being collected. Component II was present in fractions No. 56—72, which were evaporated to a thick syrup which was then dissolved in methanol. After evaporation, the residue (in water, 30 ml.) was filtered several times through kieselguhr to eliminate finely dispersed cellulose, evaporated to a syrup, and tested by paper chromatography. No trace of the above-noted contaminants could be detected. Repeated attempts to crystallise Component II failed. The syrup was ground with dry acetone and filtered, and the solid dried to constant weight under reduced pressure at 70°, giving 1·37 g., $[\alpha]_D^{20} = +22{\cdot}2°$ (*c*, 2·34) (Found: C, 42·3; H, 6·6. $C_{18}H_{32}O_{16}$ requires C, 42·9; H, 6·4%). The substance was very soluble in both water and methanol and was non-reducing to Fehling's solution and ammoniacal silver nitrate under mild conditions. It was converted into a mixture of glucose and fructose by yeast invertase or dilute acid. On complete hydrolysis by yeast invertase, $[\alpha]_D^{20}$ of the mixture agreed with that of a mixture of two parts of fructose and one of glucose.

Methylation of the Trisaccharide.—This (711 mg.) was methylated as for kestose (Albon *et al., loc. cit.*), one methyl sulphate and three methyl iodide treatments being given. The final *product*, a colourless syrup (730 mg.; 78·7%—average loss per methylation ~5%, had n_D^{20} 1·4616) (decrease for rise of 1°, 0·00034), $[\alpha]_D^{20}$ −28·0° (*c*, 9·6) (Found: C, 53·1; H, 8·4; OMe, 51·1. $C_{29}H_{54}O_{16}$ requires C, 52·9; H, 8·2; OMe, 51·8%).

Hydrolysis Products of the Methylated Trisaccharide (cf. Albon *et al., loc. cit.*).—The methyl derivative (410 mg.) was heated in 0·05N-sulphuric acid (25 ml.) at 100° until a constant $[\alpha]_D^{20}$ was observed (*l*, 4). The value of +46·4° was higher than that found for kestose and the isometric trisaccharide synthesised by mould invertase (Bacon and Bell, *loc. cit.*) and indicated that the hydrolysis mixture had a different composition. From the evidence obtained later, +46·4° agrees exactly with a mixture of two moles of 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose and one of 2 : 3 : 4-tri-*O*-methyl-D-glucose (3 : 4 : 6-tri-*O*-methyl-D-fructose and its 1 : 3 : 4 : 6-tetramethyl homologue have, of course, closely similar $[\alpha]_D$'s).

Chromatography of the mixed hydrolysis products (350 mg.) on a silica column gave two clear-cut fractions. The toluene eluate (240 mg.) had $[\alpha]_D^{20}$ +35·2°, n_D^{20} 1·4518. Paper chromatography showed that the main component was a hexulose running exactly as 1 : 3 : 4 : 6-tetra-*O*-methylfructose but that a small amount of an aldose, running exactly as 2 : 3 : 4 : 6-tetra-*O*-methylglucose, was also present. The $[\alpha]_D^{20}$ and n_D^{20} values noted above agree with a 9 : 1 mixture of these two sugars. The tetramethylfructose was identified as the crystalline " tetramethylfructofuronamide " (Haworth, Hirst, and Nicholson, *J.*, 1927, 1513).

The chloroform–butanol eluate (110 mg.) from the column consisted chiefly of an aldose running identically with 2 : 3 : 4-tri-*O*-methylglucose, and contaminated with a hexulose running identically with 3 : 4 : 6-tri-*O*-methylfructose. The $[\alpha]_D^{20}$ and n_D^{20} values of this fraction (+74° and 1·4690) correspond to an approximately 9 : 1 mixture of the aforementioned sugars. The structure of the trimethylaldose was confirmed by the preparation from this fraction of the aniline derivative of 2 : 3 : 4-tri-*O*-methyl-D-glucose (Peat, Schluchterer, and Stacey, *J.*, 1939, 581), m. p. 148—149° (from ether) not depressed on admixture with the derivative prepared from chromatographically pure sugar.

Thanks are offered to Mr. H. C. S. de Whalley (Director of Research, Tate & Lyle Ltd.), for advice and interest, Mr. R. E. Runeckles for some of the polarimetric work, and the Directors of Tate & Lyle Ltd. for permission to publish those results obtained in their Laboratory. The authors are grateful to Dr. G. D. Greville (Cambridge) for a gift of material.

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[*Received, January 15th*, 1954.]