516. *A New Trisaccharide produced from Sucrose by Mould Invertase.*

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A non-reducing trisaccharide has been isolated from the products of action of "Takadiastase" (a commercial mould-enzyme preparation) on sucrose. By methylation and analysis of the methylated sugars formed on hydrolysis the following structure has been adduced: *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2) β-D-fructofuranoside. It appears to be formed by enzymic transfer of a β-fructofuranosyl radical to sucrose.

It was found by Edelman (Thesis, Sheffield, 1950), and confirmed by Bealing and Bacon (*Biochem. J.*, 1951, **49**, lxxv), that "Takadiastase," a commercial enzyme preparation probably made from *Aspergillus oryzae*, produces a series of oligosaccharides from sucrose. This action has been found with extracts of all other moulds examined (Wallenfels and Bernt, *Angew. Chem.*, 1952, **64**, 28; Bealing and Bacon, *Biochem. J.*, 1953, **53**, 277), and Pazur (*J. Biol. Chem.*, 1952, **199**, 217) described the partial purification from extracts of *A. oryzae* of a "transfructosidase," which is probably the enzyme responsible. Bealing (*Biochem. J.*, 1953, **55**, 77; Thesis, Sheffield, 1952) put forward the view that this enzyme is, in fact, that hitherto described as "mould invertase," or "glucosaccharase," and that it acts by transferring β-fructofuranosyl radicals from sucrose either to suitable organic acceptors such as sucrose itself, or to water.

Pazur (*loc. cit.*) from a study of R_F values of partial hydrolysis products of inulin has designated as "1-inulobiosyl-D-glucose" a trisaccharide fraction ($[\alpha]_D$ +17° in H_2O) isolated by "large-scale filter paper chromatography" from the products of action of his transfructosidase on sucrose.

An adaptation of the chromatographic method of Whistler and Durso (*J. Amer. Chem. Soc.*, 1950, **72**, 677) suggested to one of us (J. S. D. B.) by Dr. A. J. P. Martin, F.R.S., of the National Institute for Medical Research, Mill Hill, revealed that the trisaccharide fraction formed from sucrose by "Takadiastase" consists of at least two components. One of these has been isolated in quantities sufficient for a structural study by methylation.

The essential feature of the modified chromatographic procedure is development by a continuously changing solvent in place of elution by stepwise increase in ethanol concentration. A similar procedure has been developed independently by Alm, Williams, and Tiselius (*Acta Chem. Scand.*, 1952, **6**, 826) and named by them "gradient elution."

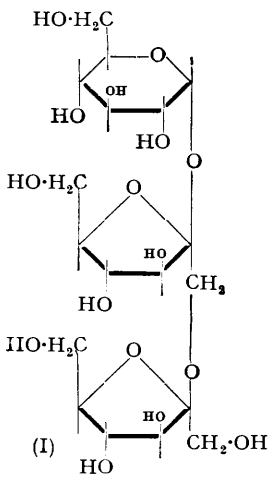
The application of the modified technique has shown that components "α" and "β" (cf. Bealing and Bacon, 1953, *loc. cit.*) each consist of at least two substances; these have been designated, in the order in which they emerge from the column, "α_1", "β_1", "α_2", and "β_2". In each case the major component emerges first. The trisaccharide referred to in this paper is "α_1", the only one of these substances readily obtained in the pure state. It was not crystallised, but was homogeneous by paper chromatography, as well as by the technique of its isolation. The yields of partially methylated sugars derived from it further suggest homogeneity.

All samples were substantially non-reducing. Analyses of ketose and reducing sugar after complete hydrolysis by yeast invertase or dilute acid, showed a ketose content consistently somewhat higher than that to be expected for a trisaccharide consisting of one glucose and two fructose residues: 5 samples gave ratios (ketose/total reducing sugar) ranging from 0·69 to 0·72.

The $[\alpha]_D$ in water of 10 samples ranged from +25·6° to +28·0° when calculated from the dry weight of syrup; calculations based on the content of reducing sugar gave values lying between +30·5° and +32·6°.

No fractions examined gave a blue, green, or yellow-green colour with diazouracil under the conditions described by Raybin (*J. Amer. Chem. Soc.*, 1933, **55**, 2603). A sample of kestose (kindly supplied by Mr. P. H. Blanchard) also gave a negative result, in contrast to the report of Albon, Bell, Blanchard, Gross, and Rundell (*J.*, 1953, 24). However, the reaction underlying this test is completely unknown, as are the factors influencing it.

The syrup was four times methylated by the procedure described for kestose [*O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(6→2) β-D-fructofuranoside] (Albon *et al.*, *loc. cit.*), to give a final yield of 59% of undeca-*O*-methyl-trisaccharide. This on hydrolysis gave 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose, 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, and 3 : 4 : 6-tri-*O*-methyl-D-fructose in equimolar proportions. From the ease of hydrolysis by dilute acid, and by yeast invertase preparations free from α-glucosidase, the glucose radical was assumed to be combined as in sucrose. After the substance had been hydrolysed slowly by acetic acid at 40° paper chromatography showed that fructose was liberated more rapidly than glucose, and a non-reducing substance having the R_F value of sucrose was produced. The whole disaccharide fraction from this partial hydrolysate had an $[\alpha]_D$, calculated from its ketose content, consistent with the assumption that it contained only sucrose, and yielded crystalline sucrose from aqueous-ethanol solution.



(I)

The tentative structure assigned to the trisaccharide is therefore *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(1→2) β-D-fructofuranoside (I), the β-linking being deduced ultimately from the accepted specificity of yeast invertase (cf. Adams, Richtmyer, and Hudson, *J. Amer. Chem. Soc.*, 1943, **65**, 136).

Our methylation studies therefore confirm the ideas of Pazur (*loc. cit.*), and further suggest that the trisaccharide is identical with that present in the artichoke tuber (cf.Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114; Dedonder, *Bull. Soc. Chim. biol.*, 1952, **34**, 144). However, the $[\alpha]_D$ of our substance is higher than that recorded by other authors: Pazur (*loc. cit.*) gives +17° for his trisaccharide, and Dedonder (*loc. cit.*) +23° for "glucofructosane B" from the artichoke. A single preparation of the latter substance by the gradient-elution technique had $[\alpha]_D$ +28°; the artichoke extract apparently contains only a single trisaccharide. The lower value given by Dedonder could be due to some impurity in his preparation, but the much lower figure given by Pazur may be due to the presence of "α_2", the $[\alpha]_D$ of which is not known, or possibly of a reducing trisaccharide formed entirely of fructose, that might arise by transfructosidation to free fructose.

The mould trisaccharide described here, like kestose (Albon *et al.*, *loc. cit.*), presumably arises by fructosyl transfer to sucrose. A satisfactory assessment of the differences in specificity between the mould enzyme and yeast invertase must await the characterisation of components I, II, and V of the yeast-invertase reaction mixture (cf. White and Secor, *Arch. Biochem. Biophys.*, 1952, **36**, 490).

Experimental

Specific rotations were determined in water, in a 2-dm. tube unless otherwise noted.

Action of "Takadiastase" on Sucrose.—A solution made by dissolving "Takadiastase" (Parke Davis & Co.) (5·5 g.) in water (25 ml.) was dialysed in Visking synthetic cellulose casing (John Crampton & Co. Ltd., Wythenshawe, Manchester) against running tap-water for 2 days, 5 ml. of toluene being added as a preservative. The dialysed solution was allowed to act on sucrose (20 g.) dissolved in water (80 ml.) and 0·2M-phosphate buffer (pH 7·0) (2 ml.) at room temperature for 20 hr. The enzyme was then inactivated by boiling and the solution stored at −20°. The preparation used for methylation was isolated from this solution. Other reaction mixtures were prepared and yielded essentially similar trisaccharide fractions.

Chromatography.—This was based on Whistler and Durso's method (*loc. cit.*). Activated charcoal (British Drug Houses Ltd.) and active carbon No. 130 (Sutcliffe, Speakman & Co. Ltd., Leigh, Lancs.) were used with "Celite No. 535" (Johns Manville Co. Ltd., London, S.W.1.). The charcoals were similar in their properties; the former preparation appeared to have a higher resolving power for sugar mixtures, but the latter was preferred because it gave a neutral effluent.

A typical preparation was as follows: a mixture of active carbon No. 130 (40 g.) and "Celite No. 535" (40 g.) was packed wet (350 ml. of water) into a glass tube, forming a column 28 mm. in diam. and about 400 mm. high. The enzyme reaction mixture (10 ml.) was allowed

to soak into the top of the column and was washed in with water (15 ml.). The top of the column was then attached through a narrow siphon tube to a closed 1-l. conical flask full of water (1000 ml.), into which 50% aqueous ethanol (v/v) was allowed to drip to replace the fluid which entered the charcoal–Celite column; the contents of the flask were stirred by a magnetic stirrer operating for 1 min. in each 5-min. period (cf. Alm *et al.*, *loc. cit.*). In this way the concentration of ethanol in the solvent entering the column increased in a reproducible manner. Monosaccharide, mainly glucose, emerged after 250 ml. of effluent had passed, disaccharide, mainly sucrose, after 440 ml., and " α_1 " after 560 ml.; the sucrose and trisaccharide fractions overlapped to a slight extent, " α_1 " and " β_1 " to a somewhat greater extent, but the greater part of " α_1 " was apparently homogeneous. The concentrations of sugars in the effluent were such that 5 μl. contained an ample amount for examination by paper chromatography. By two such operations about 900 mg. were obtained for methylation.

The optical rotation of such fractions was read in 2—5% solution, and the same solution analysed by the methods described by Bacon and Edelman (*loc. cit.*).

The material was converted into a mixture of glucose and fructose by the action of yeast invertase (B.D.H. " Invertase Concentrate ") or of dilute acid. No intermediates were detected in enzymic hydrolyses, but on addition of acetic acid (20% by vol.) at 40° appreciable amounts of a non-reducing disaccharide were formed. After hydrolysis for 6 hr. in this manner 68 mg. of a disaccharide fraction were isolated by the gradient-elution technique from 0·5 g. of trisaccharide. 48 Mg. of the fraction (ketose content, 22·5 mg.) in 3 ml. of water had a rotation of $+1·88°$; on the assumption that it is sucrose the rotation corresponds to $[\alpha]_D$ $+66°$ (sucrose has $[\alpha]_D$ $+66·5°$). The fraction was dissolved in 75% aqueous ethanol (3 ml.) and treated with charcoal (30 mg.). The filtrate was taken to dryness and dissolved in 50% aqueous ethanol (0·25 ml.). Absolute ethanol (2 ml.) was added and the solution set aside. There was a growth of crystals, m. p. 186° (mixed m. p. with authentic sucrose, 184—185°); 5·6 mg. treated with 1·2 mg. of diazouracil in 0·5 ml. of N-sodium hydroxide at 10° gave the deep blue-green colour typical of sucrose.

Tests were carried out as described by Raybin (*loc. cit.*) on 50-mg. samples of the trisaccharide, and also on 10-mg. samples with one-fifth quantities of reagents. In no case was any green colour observed. Sucrose and raffinose gave typical blue-green colours, and melezitose and kestose a brownish-yellow colour, similar to that given by the mould trisaccharide.

Methylation of the Trisaccharide.—880 Mg. were treated as described for kestose (Albon *et al.*, *loc. cit.*); 680 mg. (59%) of *undeca-O-methyl-trisaccharide* were obtained (average loss per methylation ~10%) as a syrup having n_D^{20} 1·4613 (decrement for rise in temp. of 1°, 0·00034) and $[\alpha]_D^{20}$ $+27·9°$ (*c*, 2·5) (Found: C, 53·4; H, 8·4; OMe, 51·2. $C_{29}H_{54}O_{16}$ requires C, 53·2; H, 8·05; OMe, 51·8%).

Hydrolysis Products of the Methylated Trisaccharide (cf. Albon *et al.*, *loc. cit.*).—320 Mg. were heated in 0·05N-sulphuric acid (25 ml.) at 100° till a constant $[\alpha]_D^{20}$ of $+44·6°$ was observed (*l*, 4). This rotation corresponds closely to that expected from an equimolar mixture of 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose, 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, and 3 : 4 : 6-tri-*O*-methyl-D-fructose (cf. Bell, *J.*, 1953, 1231). Paper chromatography showed the presence of both of the first two sugars along with that of a tri-*O*-methylketose moving at the same rate as the above-mentioned fructose derivative.

Silica column chromatography of the mixed hydrolysis products (320 mg.) gave two clear-cut fractions. The toluene eluate (190 mg.) had $[\alpha]_D^{20}$ $+56·7°$ (calc., $+55·9°$); further examination by the procedure previously used with methylated kestose gave crystalline 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose (m. p. and mixed m. p. 95—98°) and a syrup having the chromatographic properties of 1 : 3 : 4 : 6-tetra-*O*-methylfructose. The methanolic eluate of the column yielded 99 mg. (97%) of a syrup (OMe, 41·2%), $[\alpha]_D^{20}$ $+29·3°$, n_D^{20} 1·4652. This was proved to be 3 : 4 : 6-tri-*O*-methyl-D-fructose, since (*a*) 24·63 mg. oxidised by IO_4^- (Bell, Palmer, and Johns, *J.*, 1949, 1536; Bell and Palmer, *J.*, 1952, 3763) gave 31·93 mg. (0·97 mol.) of formaldehyde–dimedone compound, m. p. 190—192° (corr.) and (*b*) the sugar yielded 3 : 4 : 6-tri-*O*-methyl-D-glucose phenylosazone, m. p. and mixed m. p. 125—126°.

This work was done with the technical assistance of Miss B. Dickinson. We thank Dr. Martin for his suggestion about the chromatographic procedure, and Miss J. Cawkwell for assistance during the early stages.

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