Preparation and Characterization of the Isomaltodextrins

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(*Received* 20 *February* 1957)

In the course of studies on the amylopectin-debranching enzyme, R-enzyme (Hobson, Whelan & Peat, 1951; Peat, Whelan, Hobson & Thomas, 1954), it became necessary to prepare individual members of the series of glucose oligosaccharides containing the amylopectin-type branch linkage as the sole polymeric bond, i.e. the isomaltodextrins. The opportunity to do this was provided by the acquisition of a *Leuconostoc mesenteroides* dextran containing a very high proportion (96%, determined by periodate oxidation) of α-1:6-linkages. Acid-catalysed fragmentation of this polyglucose to an appropriate degree should provide a mixture of small isomaltodextrins, capable of being fractionated by charcoal chromatography. This expectation has been realized and the individual members within the series isomaltose–isomaltohexaose have been isolated and characterized. The last three members, isomalto-tetraose, -pentaose and -hexaose, have not previously been described, except in terms of R_F value.

The oligosaccharides described have been used in studies of the inhibition of precipitation of dextran by human antidextran (Kabat, 1956).

RESULTS

Hydrolysis of the dextran

The dextran was hydrolysed in dilute sulphuric acid until the apparent conversion into glucose was 52%, a value chosen on the basis of the Kuhn (1930) formula as appropriate for the maximum yield of tri- to penta-saccharides. Fractionation and refractionation on charcoal–Celite led to the separation of glucose, as the only monosaccharide component, and five amorphous sugars of decreasing R_F value, each of which migrated as a single zone during paper-chromatographic fractionation. The oligosaccharides were also of high purity in respect of freedom from non-carbohydrate material (Table 1).

Properties of the isomaltodextrins

Molecular weight. The molecular weights of the dextrins were determined by comparison of their copper-reducing powers, the assumptions being made that each molecule contains only one free-reducing group and that the molecular weight of the lowest member, isomaltose, is 342. The results are in excellent agreement with expectation (Table 1).

Optical rotation. The molecular rotations of the dextrins showed the expected linear relationship with degree of polymerization (Fig. 1, Whelan, Bailey & Roberts, 1953; Lindberg & McPherson, 1954; French, 1955). Glucose does not conform to this relationship, an observation already made with the maltodextrins (Whelan *et al.* 1953), the laminaridextrins (Lawley, 1955) and the gentiodextrins (Haq & Whelan, 1956), indicating that the disaccharide must be regarded as the lowest member of any such polymeric series.

Jones, Jeanes, Stringer & Tsuchiya (1956) have described the preparation and properties of the methyl α-glycosidic derivatives of isomaltose, isomalto-triose, -tetraose and -pentaose. It is to be expected that the molecular rotational increment/glucose residue in this series should be the same as in the series of free sugars. The calculated value for the glycosides is +318°; for our series the value is

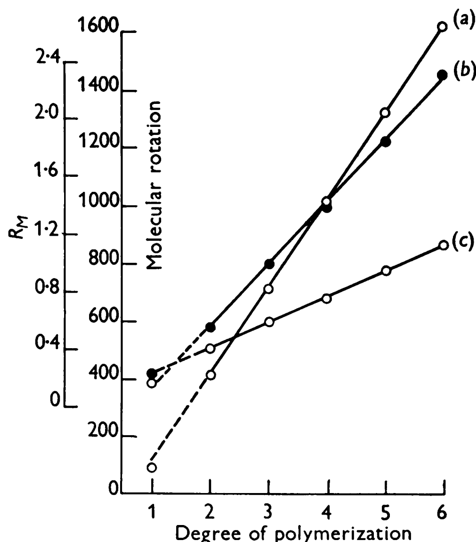

Fig. 1. Relations between degree of polymerization of the isomaltodextrins and (*a*) molecular rotation, (*b*) and (*c*) R_M values (see text) calculated from R_F values determined in butanol–pyridine–water (6:4:3, by vol.) and propanol–ethyl acetate–water (6:1:3, by vol.) respectively, by descending chromatography on Whatman no. 54 paper.

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Table 1. *Properties of the isomaltodextrins*

The yields are those from 54 g. of dextran; 21·1 g. of glucose was also obtained. Charcoal–Celite column (Whelan *et al.* 1953) 95 cm. × 5 cm. The elution volume is the volume of eluate in which the sugar appeared during refractionation by gradient elution (see Experimental section).

Substance	Elution vol. (l.)	Yield (g.)	Carbohydrate content (%)	$[\alpha]_D$ in water	Mol.wt. Found	Mol.wt. Calc.	Ester derivative m.p.	Ester derivative $[\alpha]_D$ in $CHCl_3$
Isomaltose	—	9·3	—	+122°	—	342	143·5–144·5°*	+97°
Isomaltotriose	7·0–9·2	4·0	97·1	+142†	500	504	226–227‡	+131
Isomaltotetraose	10·8–12·6	2·2	98·1	+153	672	666	176–179‡	+166
Isomaltopentaose	14·2–16·2	2·4	98·6	+160	826	828	—	—
Isomaltohexaose	17·8–18·8	0·58	95·2	+163	987	990	—	—

* Acetyl derivative; values for authentic specimens are given by Bacon & Bacon (1954).
† Jeanes *et al.* (1953) quote +145·0° for this substance; Wolfrom & Thompson (1956) quote +128°.
‡ Benzoyl derivative.

+300°. The average increments in the series of methyl $\alpha\beta$-glycosides prepared by methanolysis of a similar dextran is +367° (Scott & Senti, 1955). Possibly the large difference from the foregoing values is due to an inconstant ratio of the α- and β-forms in the series of methyl glycosides.

R_F values. The R_M values, log $(1/R_F - 1)$, of the isomaltodextrins in two solvent systems are plotted against degree of polymerization in Fig. 1. The expected linear relationships were again observed (cf. Whelan *et al.* 1953; French & Wild, 1953).

Esters. Isomaltose can be characterized as the crystalline octa-acetate but isomaltotriose acetate could not be crystallized, either by Jeanes, Wilham, Jones, Tsuchiya & Rist (1953) or by the present authors. Recourse was had to the preparation of benzoyl derivatives, and crystalline specimens of β-isomaltotriose undecabenzoate and β-isomaltotetraose tetradecabenzoate were obtained (Table 1).

DISCUSSION

Structure of the isomaltodextrins

The properties of the disaccharide and its octa-*O*-acetyl derivative were identical with those reported for isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucose]. The properties of the higher dextrins, namely molecular weights, molecular rotations and R_M values, are entirely consistent with their being members of the polyglucose series based on the repeating α-1:6-linkage and, in addition, the trisaccharide had the same specific optical rotation as that already reported for isomaltotriose (see Table 1). The structures of the tri- to hexa-saccharides are therefore established.

On the evidence that only α-1:6-linked glucose polymers were obtained from the dextran hydrolysate, it might be concluded that the original polysaccharide molecule contained only these linkages. As already mentioned, periodate oxidation indicated that 96 % of the linkages were of the 1:6-type. Recent methylation studies by American workers have, however, shown that a similar dextran, produced by the NRRL B-512 strain of *Leuconostoc mesenteroides* and having 95 % of 1:6-links (by periodate oxidation: Jeanes & Wilham, 1950; Rankin & Jeanes, 1954; Dimler, Wolff, Sloan & Rist, 1955), also contained about 5 % of 1:3-bonds (Van Cleve, Schaefer & Rist, 1956). These additional linkages apparently constitute points of branching. If such linkages were also present in our dextran it is unlikely that they would cause contaminants, in the way of 1:3-linked sugars, to be present in the purified oligosaccharides, even to the extent of only 5 %. There are three reasons for this statement: First, the 1:6-link is more resistant to acid hydrolysis than the 1:3-link. The breakdown products will therefore contain fewer than 5 % of 1:3-links. Secondly, the paper- and charcoal-chromatographic separations of isomaltose from nigerose (α-1:3-link) or laminaribiose (β-1:3-link) are very marked, and such contaminants would therefore be easy to recognize. Thirdly, in fractionation of the dextran hydrolysate the fractions representing the beginning and end of the elution of a sugar were deliberately rejected to ensure the purity of the major product. Our results do not therefore allow us to conclude anything about the structure of the polysaccharide, other than that the great majority of the polymeric linkages must be of the α-1:6-type.

Reducing powers of the isomaltodextrins

Whelan *et al.* (1953) showed that the molar-reducing power towards Somogyi (1945*a*) copper reagent in the series maltose–maltoheptaose was constant, but in order to develop the full reducing power of each maltodextrin it was necessary to heat it with the copper reagent for a period which increased with the molecular size of the dextrin. Maltose required 20 min. heating and maltoheptaose 45 min. By contrast, the isomaltodextrins

all required the same time to develop full reducing power, namely 30 min. A similar observation has been made with the 1:6-linked polymers of β-D-glucose (Haq & Whelan, 1956).

This information is relevant to the attempts that have been made to determine the molecular weights of polysaccharides by measurements of copper-reducing power. Such methods are notoriously unreliable for the large starch-type polymers containing the maltose linkage, and the results obtained by Whelan *et al.* (1953) offer an explanation of this unreliability. Isbell, Snyder, Holt & Dryden (1953) obtained good agreement between molecular weights of dextrans as measured by copper-reducing power, calibrated against isomaltose, and molecular weights measured by other methods. The present results are in agreement with these observations.

EXPERIMENTAL

Preparation of the dextran

The dextran was a purified, partly degraded specimen with an approximate weight-average molecular weight of 180 000, elaborated by the NRRL B-512 strain of *Leuconostoc mesenteroides*. This type of dextran and the organism have been described by Jeanes, Wilham & Miers (1948).

Hydrolysis of the dextran and fraction of the products

The dextran (54 g.) was dissolved in hot water (1 l.), and 6N-H_2SO_4 (277 ml.) and water were added to give 5 l. of solution, 0·33N with respect to acid. This was heated under reflux on a boiling-water bath and samples were removed at intervals for determination of copper-reducing power, as glucose (Somogyi, 1945*a*). After 10 hr., when the reducing power corresponded to 52% conversion into glucose, the solution was cooled and neutralized to pH 6–7 with NaOH solution. The hydrolysate was absorbed on a charcoal–Celite column (95 cm. × 7·3 cm.), prepared as by Whelan *et al.* (1953), and fractions (500 ml. each) were collected during elution with water and then with stepwise-increasing concentrations of ethanol in water (5, 8, 15, 20 and 50%, v/v). The optical rotations of the fractions were measured in a 4 dm. tube. Glucose (21·1 g.) appeared in the fractions corresponding to 7·5–27·5 l. of water. Ethanol (5%) was then added. Fractions corresponding to 28–36·5 l. (1·2 g.) contained a mixture of glucose and isomaltose, while fractions corresponding to 37–48 l. (10·3 g.) contained isomaltose with a trace of glucose. The higher fractions were not obtained in a sufficiently pure state, with respect to freedom from other sugars, and they were combined and refractionated on a charcoal–Celite column (95 cm. × 5 cm.) by gradient elution, 30% aqueous ethanol being fed through a constant-head device into a reservoir of water (15 l.), which in turn was connected to the column. Fractions (200 ml. each) were collected and their optical rotations determined as before. Fractions representing the peaks of optical activity were combined, evaporated to dryness, and freed from inorganic matter as by Whelan *et al.* (1953). The isomaltose was refractionated on charcoal–Celite to remove the traces of glucose, and the weights of chromatographically pure fractions are shown in Table 1.

Properties of the isomaltodextrins

Isomaltodextrin solutions used for analytical determinations were first treated with Somogyi's (1945*b*) deproteinizing reagents, as by Whelan *et al.* (1953), to remove substances interfering with reducing-power determinations.

Carbohydrate content and specific optical rotation. The carbohydrate contents of the fractions were determined by acidic hydrolysis to glucose under the conditions defined by Pirt & Whelan (1951), except that the time of hydrolysis was increased to 10 hr., experiments having shown that isomaltotriose was not completely hydrolysed after the usual 2 hr. heating period and that the reducing power of the hydrolysate gradually increased to a maximum at 10 hr. heating. The appropriate correction factor was applied for loss of glucose due to acidic degradation and reversion (Pirt & Whelan, 1951). The weight of isomaltodextrin present was calculated on the basis of its molecular size as judged by the R_M value (Fig. 1). The carbohydrate contents of the specimens are listed in Table 1.

Specific optical rotations were calculated from measurements (4 dm. tube) on isomaltodextrin solutions of which the concentrations had been determined by acidic hydrolysis.

Molecular weights. When isomaltopentaose was heated with Somogyi (1945*a*) copper reagent for times varying from 20 to 60 min. the reducing power was found to be constant between 30 and 60 min., and at 20 min. was less than 0·5% below the maximum value. A time of 30 min. was chosen and the reducing powers of isomaltodextrin solutions of known concentration were measured. The molecular weights were calculated on the assumption that the molecular weight of the disaccharide was 342 and that each dextrin contained only one free reducing group (Table 1).

Crystalline ester derivatives

Isomaltose (204 mg.) was acetylated with acetic anhydride–sodium acetate to yield the crystalline β-octaacetate (yield, 63%; Table 1). The m.p. was not depressed on admixture of the product with an authentic specimen.

Isomaltotriose (238 mg.) was dissolved in warm pyridine (5 ml.), and benzoyl chloride (1·5 ml.) was added slowly to the cooled solution, the temperature being kept below 40°, after which the stoppered vessel was shaken for 24 hr. at 0–5° and finally refluxed at 120–130° for 1 hr. The solution was poured into iced water (150 ml.), and the mixture shaken at 0° for 24 hr. and extracted with chloroform (3 × 50 ml.); the combined extracts were washed with water, dilute $NaHCO_3$, and water, then dried (Na_2SO_4). The solution was evaporated to a syrup which was decolorized in ethanolic solution with charcoal and left to crystallize. Recrystallization from ethanol was unsuccessful but several recrystallizations of the coarse white needles were made from ethanol–acetone (1:1, v/v). The yield was 308 mg. (40%) of *β-isomaltotriose undecabenzoate* (Table 1) (Found: C, 68·75; H, 4·6. $C_{95}H_{76}O_{27}$ requires C, 69·2; H, 4·6%).

Isomaltotetraose (200 mg.) was treated similarly and an amorphous product was obtained from ethanol–acetone. This was crystallized from ethanol–benzene (1:1, v/v), to which water had been added until turbidity appeared, and cleared by the addition of ethanol. On slow evaporation at room temperature fine colourless needles were deposited, together with some amorphous material. The same solvent

system was used for recrystallization. The yield was 432 mg. (69%) and the properties of the *β-isomaltotetraose tetradecabenzoate* are listed in Table 1 (Found: C, 68·6; H, 4·5. $C_{122}H_{98}O_{35}$ requires C, 69·0; H, 4·65%).

Attempts to prepare a crystalline octabenzoate of isomaltose were unsuccessful.

SUMMARY

1. A dextran synthesized by *Leuconostoc mesenteroides* (strain NRRL B-512) has been partly hydrolysed with acid and the products were fractionated on charcoal–Celite.

2. Glucose was the only monosaccharide product and the di- to hexa-saccharide fractions each contained a single component.

3. The oligosaccharides have been characterized as the lower members of the isomaltodextrin series and new crystalline derivatives of the tri- and tetra-saccharides have been prepared.

4. Some observations on the copper-reducing powers of glucose oligosaccharides are recorded.

We are grateful to Dr A. T. James for the gift of the dextran, and to Professor Stanley Peat, F.R.S., for his interest and encouragement.

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The Determination of 10–100 μmg. Quantities of Hexosamine

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(*Received* 5 *November* 1956)

The most sensitive methods for the estimation of hexosamine described so far are those of Elson & Morgan (1933) as modified by Blix (1948), and of Dische & Borenfreund (1950). During the course of investigations in this laboratory it became necessary to determine as little as 10 μmg. of hexosamine in 0·2–0·5 μl. samples of the endolymph of the inner ear. Ultramicro modifications of the above methods allow at best quantities of 40 μmg. of hexosamine to be determined, and were therefore not sufficiently sensitive.

The present paper describes a more sensitive and more specific method for the determination of hexosamine, which allows 10–100 μmg. to be determined. The bound hexosamine is liberated by acid hydrolysis, and separated from sugars and amino acids by treatment with an ion-exchange resin from which it is eluted with hydrochloric acid. The hexosamine is then deaminated by treatment with sodium nitrite in acetic acid to give hydroxymethyldihydroxytetrahydrofurfuraldehyde (2:5-anhydrosugars) after a Walden inversion at $C_{(2)}$ of the hexosamine. Degradation of these stable hydrofuran derivatives by hydrochloric acid yields laevulic and formic acids, through the intermediates hydroxymethylfurfuraldehyde and hydroxylaevulic aldehyde (Fig. 1) (Newth, 1951). Reaction between 2:5-anhydrosugars and various