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# STRUCTURE OF THE D-FRUCTAN ISOLATED FROM GARLIC (*Allium sativum*) BULBS*

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ABSTRACT

Extraction of defatted garlic bulbs with hot water yielded a mixture of polysaccharides containing pectic acid, a D-galactan, and a fructan component. The pectic acid was partially removed as calcium pectate, and the galactan-enriched portion was separated by fractional precipitation with alcohol; on concentration and several fractionations, the supernatant liquor furnished the fructan component, which contained fructose (94.4%) and glucose (4.3%). Methanolysis and hydrolysis of the permethylated fructan gave (*a*) 1,3,4,6-tetra-*O*-methyl-D-fructose, (*b*) 2,3,4,6-tetra-*O*-methyl-D-glucose, (*c*) 2,4,6-tri-*O*-methyl-D-glucose, and (*d*) 3,4,6-tri-*O*-methyl-D-fructose in the ratio $(a + b):(d) = 1:20.3$. On periodate oxidation, the fructan reduced one molar equivalent of the oxidant per hexosyl residue, and liberated one molar equivalent of formic acid per 51 hexosyl residues. On Smith degradation, the major product was glycerol, and ~2% of the glucose survived. From these results, and from the fact that the fructan is hydrolyzed by β-D-fructofuranosidase, a linear, inulin-type of structure is suggested for it.

## INTRODUCTION

It was reported[1] earlier that extraction of defatted garlic bulbs with hot water yields a mixture (B) of polysaccharides that contains D-galacturonic acid (as pectic acid), D-galactose (as a galactan), and D-fructose (as the third major constituent); in addition, there are small proportions of D-glucose, L-arabinose, and L-rhamnose. Fractionation of B with calcium chloride solution removed a part of the pectic acid, and the supernatant liquor, on concentration and fractional precipitation, furnished polysaccharides $B_1$ and $B_2$. $B_1$ was the galactan-enriched fraction, and $B_2$ contained mainly fructose, along with a small proportion of glucose (total hexose, 94.2%). Traces of galactose (1.1%), galacturonic acid (1.6%), and arabinose (2.7%) were also present in $B_2$.

*Characterization of the Polysaccharides of Garlic *(Allium sativum)* Bulbs, Part II. For Part I, see ref. 1.

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In order to isolate the fructan component in pure form, $B_2$ was extracted with aqueous 70% ethanol, and the extract was concentrated, and the polysaccharide precipitated with ethanol. The precipitate was fractionally reprecipitated (4 times) from its aqueous solution with dry ethanol, to afford polysaccharide $B_9$ having $[\alpha]_{589.5}$ −42.3°, and a total hexose content of 98.7%, of which fructose constituted 94.4% and the rest (4.3%) was glucose. The value of the specific rotation did not change on further fractional precipitation.

On subjecting polysaccharide $B_9$ to high-voltage electrophoresis, and developing with the benzidine–periodate reagent[12], it was resolved into two partially merged zones — a blue zone (polysaccharide $B_{10}$) and a white zone (polysaccharide $B_{11}$) — their centers moving 4.7 and 2.5 cm, respectively, from the base line towards the cathode. On the same paper, D-glucose moved 10.8 cm towards the anode. The resolution of $B_9$ into two components apparently shows some heterogeneity in the fructan. To settle this question, and also to determine whether the small proportion of glucose present in $B_9$ is chemically bound to the fructan, sufficient quantities of $B_{10}$ and $B_{11}$ were isolated by repeated electrophoresis of $B_9$, and the products were analyzed (see Table I). The very close values of their specific rotations, and the similar glucose to fructose ratios of $B_9$, $B_{10}$, and $B_{11}$ indicated that the three polysaccharides have practically the same chemical composition. The apparent electrophoretic heterogeneity is, therefore, likely to be due to polydispersity.

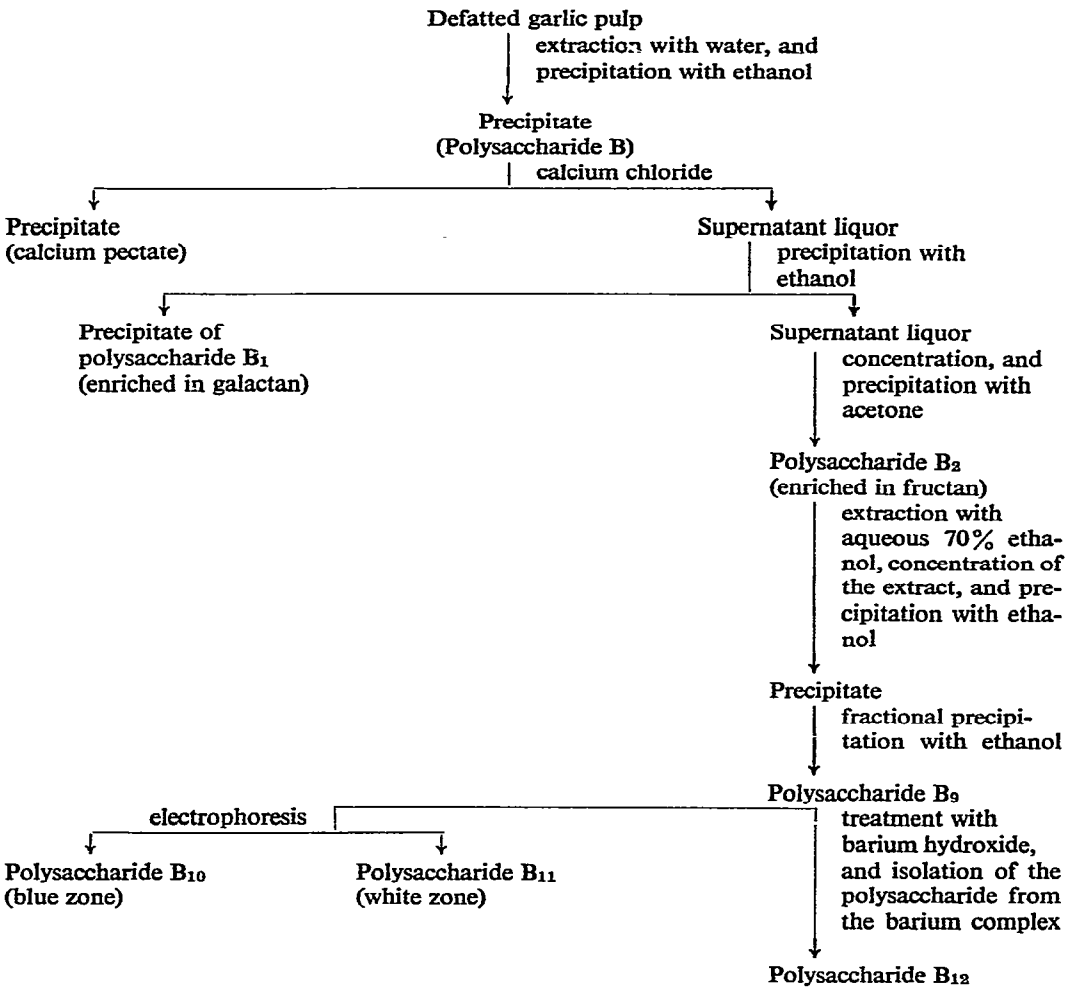
TABLE I

CHARACTERISTICS OF POLYSACCHARIDES $B_9$, $B_{10}$, $B_{11}$, AND $B_{12}$

| *Polysaccharide fraction* | $[\alpha]_{589.5}$ *(in water; degrees)* | *Composition* | | |
|---|---|---|---|---|
| | | *Total hexose (%)* | *Fructose (%)* | *Ratio[a] of glucose:fructose* |
| $B_9$ | −42.3 | 98.7 | 94.4 | 1:18.7 |
| $B_{10}$ | −42.6 | 98.1 | 94.0 | 1:18.1 |
| $B_{11}$ | −41.8 | 97.9 | 94.6 | 1:18.5 |
| $B_{12}$ | −42.4 | 98.6 | 93.8 | 1:19.0 |

[a]Results of g.l.c. (column I, 170°) of the hydrolyzates as per(trimethylsilyl) derivatives; the same conditions of hydrolysis (*viz.*, 1% oxalic acid for 1 h at 100°) were used in all cases.

On treatment with barium hydroxide solution, $B_9$ gave a precipitate (a similar observation was reported by Belval[2] *et al.*) that, on decomposition with carbon dioxide in aqueous medium, and de-ionization and lyophilization of the resulting solution, afforded polysaccharide $B_{12}$, which had a specific rotation, and a glucose to fructose ratio, almost identical to those of the parent compound (see Table I), supporting the conclusions that the electrophoretic heterogeneity is due to polydispersity, and that the glucose portion is probably bound chemically to the fructan. The isolation of the polysaccharides ($B_9$ through $B_{12}$) is summarized in Scheme 1.




Scheme 1. Fractionation of the polysaccharides of garlic bulbs, and isolation of the fructan component.

On separate treatment with *alpha*- and *beta*-amylase, polysaccharide $B_9$ did not release any reducing sugar, whereas a mixture of $B_9$ and amylose with either enzyme released reducing sugars within five minutes of incubation, indicating that the glucose portions are not joined by α-D-(1→4)-linkages. On the other hand, on treatment with β-D-fructofuranosidase (yeast invertase), $B_9$ released only fructose (at a very low rate, compared to that with sucrose as the substrate). This confirmed the presence of β-D-fructofuranose residues in $B_9$, although the slowness of the release is not yet understood.

From these findings, it is now reasonable to believe that the polysaccharide preparations ($B_9$ through $B_{12}$) are essentially the same, and homogeneous, containing the glucose portion chemically bound in them.

Polysaccharide $B_9$ was completely acetylated, and the product was fractionated

by extraction with methanol, to afford the acetylated polysaccharides $B_{13}$ (soluble in methanol) and $B_{14}$ (insoluble in methanol). $B_{14}$ had $[\alpha]_{589.5}$ $-28.3°$, and showed no absorption band in the hydroxyl region of its i.r. spectrum. Both $B_{13}$ ($[\alpha]_{589.5}$ $-28.7°$) and $B_{14}$ had to be solubilized with 70% sulfuric acid before hydrolysis. G.l.c. of the hydrolyzates as the per(trimethylsilyl) derivatives showed that $B_{13}$ and $B_{14}$ contain fructose and glucose in the ratios of 11.1:1.0 and 13.8:1.0, respectively. Under similar conditions of hydrolysis, $B_9$ furnished fructose and glucose in the ratio of 13.6:1.0. These results may lack quantitative significance, because the conditions of hydrolysis were drastic for fructosyl residues, which, if unacetylated, are[3] substantially decomposed by mineral acids; however, the major fraction ($B_{14}$) has a composition comparable to that of the parent compound ($B_9$). The appearance of a small proportion of $B_{13}$ may be ascribed to polydispersity, or incomplete acetylation, or both. Also, the presence of glucose in both $B_{13}$ and $B_{14}$ is in conformity with the conclusion that the glucose units are chemically bound to the fructan.

To determine the nature of the linkages between the monosaccharide units of the fructan, $B_{14}$ was fully methylated (Hamilton and Kircher[4] and Purdie and Irvine[5]), to afford a semisolid, brown mass having $[\alpha]_{589.5}$ $-44.2°$ and OMe 43.6%; its infrared spectrum showed no hydroxyl band. The permethylated polysaccharide was fractionally precipitated, to give fraction $b_1$ as a dark-colored, sticky material having $[\alpha]_{589.5}$ $-41.6°$ and OMe 42.5%, and fraction $b_2$ as orange flakes having $[\alpha]_{589.5}$ $-46.4°$ and OMe 44.1%.

G.l.c. of the methyl glycosides $h_1$, prepared by methanolysis of $b_2$, furnished two major peaks, corresponding to methyl 3,4,6-tri-*O*-methyl-fructosides, and minor peaks corresponding to methyl 1,3,4,6-tetra-*O*-methyl-fructosides, methyl 2,3,4,6-tetra-*O*-methyl-glucosides, and methyl 2,4,6-tri-*O*-methyl-glucosides. In addition, some unidentified peaks were detected (see Table IVa). The ratio of tri-*O*-methyl-fructose to total tetra-*O*-methylated sugars was 14.9:1 (see Table II); however, as mentioned earlier, this ratio may be inaccurate, because of the susceptibility[3] of fructosyl residues to mineral acids, and some of the unidentified peaks may be those of methylated difructan dianhydride, reported[6] to be formed under similar conditions, that would affect the ratio.

TABLE II

IDENTIFICATION OF METHYLATED SUGARS OBTAINED FROM $b_2$ UNDER DIFFERENT CONDITIONS

| *Condition* | *Methylated sugars detected* | *Ratio of* $(a+b):d$ |
|---|---|---|
| Methanolysis with dry methanolic hydrogen chloride (0.25%) ($h_1$) | (a) 1,3,4,6-tetra-*O*-methylfructose<br>(b) 2,3,4,6-tetra-*O*-methylglucose<br>(c) 2,4,6-tri-*O*-methylglucose<br>(d) 3,4,6-tri-*O*-methylfructose | 1:14.9 |
| Hydrolysis with 1% oxalic acid in 70% aqueous methanol ($h_2$) | (a) 1,3,4,6-tetra-*O*-methylfructose<br>(b) 2,3,4,6-tetra-*O*-methylglucose<br>(c) 2,4,6-tri-*O*-methylglucose<br>(d) 3,4,6-tri-*O*-methylfructose | 1:20.3 |

Demethylation[7] of $h_1$ furnished only glucose (paper chromatogram), obviously because of complete decomposition of the fructose portion by hydrobromic acid. Hydrolysis of $h_1$ with oxalic acid[3] yielded a mixture of the methylated sugars which, on chromatography (solvent *B* and staining reagent *b*) furnished a brownish-yellow spot ($R_G$* 0.93), corresponding to 2,4,6-tri-*O*-methylglucose, and a pink spot ($R_G$ 1.00) corresponding to 2,3,4,6-tetra-*O*-methylglucose; the former spot was somewhat elongated, probably due to the presence of some of the methylated difructan derivative mentioned earlier.

To avoid the degradative effect of mineral acids, another portion of $b_2$ was hydrolyzed with oxalic acid[3]. The results of paper chromatography of the hydrolyzate, $h_2$, agreed with those for the oxalic acid hydrolyzate obtained from $h_1$. After conversion of $h_2$ into the mixture of methyl glycosides, g.l.c. corroborated the earlier findings (see Table II), but the ratio of tri-*O*-methyl to total tetra-*O*-methyl sugars was now 20.3:1, and the proportion of unidentified peaks was much smaller than for $h_1$.

The portion ($f_1$) of $h_2$ that responded positively to staining reagent *d* was isolated by preparative paper-chromatography, and identified as 3,4,6-tri-*O*-methyl-D-fructose by its methoxyl content and specific rotation, and by the g.l.c. data. Also, on treatment with alkali and demethylation of the product, $f_1$ furnished a mixture of glucose and mannose. As epimerization requires that O-1 and O-2 of the methylated fructose ($f_1$) be free, and as O-5 is involved in the furanose ring, the only positions available for methyl groups in $f_1$ are O-3, O-4, and O-6. The red stain formed by $f_1$ with reagent *d* also substantiates this identification[8].

From these results, certain conclusions may be drawn regarding the structure of the fructan. The absence of any dimethyl sugar indicates that the fructan molecules are linear, and as the major tri-*O*-methyl sugar is 3,4,6-tri-*O*-methyl-D-fructose, the nonterminal D-fructosyl residues are linked (2→1).

Formation of 1,3,4,6-tetra-*O*-methyl-D-fructose and 2,3,4,6-tetra-*O*-methyl-D-glucose shows that the terminal unit at one end of the molecule is a D-fructosyl group, and at the other end, a D-glucosyl group. The presence of small peaks for the methyl 2,4,6-tri-*O*-methyl-D-glucosides in the g.l.c. profile for both $h_1$ and $h_2$ indicates that some D-glucosyl residues are linked (1→3) within the molecule.

On periodate oxidation (which became constant in 12 h), both $B_9$ and $B_{11}$ reduced one molar equivalent of the oxidant per hexosyl residue. Smith degradation of $B_9$ and $B_{11}$ furnished glycerol (major product) and glucose (minor product). Interestingly, the proportion of surviving glucose (1.83%) in Smith-degraded $B_9$ was about half of the original content (4.3%) of glucose. A second Smith-degradation caused little change in the glucose content (1.74%). From these results, it is clear that about half of the D-glucosyl residues are (1→3)-linked within the molecule, and this accounts for the formation of 2,4,6-tri-*O*-methyl-D-glucose. Therefore, the remaining, oxidizable half of the D-glucose must have constituted the other, nonreducing end

*$R_G$ values are with respect to 2,3,4,6-tetra-*O*-methyl-D-glucose as unity.

of the linear fructan molecule. The formation of glycerol as the major product, and complete oxidation of the D-fructosyl residues, are consistent with the conclusion that the D-fructosyl residues are (2→1)-linked.

In a separate experiment, periodate oxidation of polysaccharide $B_9$ liberated one molar equivalent of formic acid per 51 hexosyl residues. As none of the D-fructosyl residues (including the terminal group) can liberate any formic acid, the formic acid must have originated from the D-glucosyl group of the other terminal. Hence, considering the percentage (~2%) of the terminal D-glucosyl groups, it is apparent that there is one terminal D-glucosyl group per 51 hexosyl residues. In other words, there are two glucose units per molecule of the fructan — one at the protected "reducing" terminal, probably as in sucrose, and the other somewhere within the molecule. Based on these results, structure **1** may be assigned to the fructan, whose average degree of polymerization (d.p.) is 51, as calculated from the formic acid data. The d.p. could also have been calculated from the methylation data, but greater reliability accrues to the formic acid data, for reasons already mentioned.

Fru*f*-(2→1)-[Fru*f*]$_{48-x}$-(2→3)-Glc*p*-(1→1)-[Fru*f*]$_x$-(2→1)-Glc*p*

**1**

As the values of the specific rotation of the fructan and of its acetylated and methylated derivatives are negative, and as the fructan is hydrolyzed by β-D-fructofuranosidase, the D-fructosyl residues are presumably joined mainly by β-D-linkages. The anomeric nature of the linkage of the D-glucose units cannot be ascertained from the foregoing data. It may be mentioned that structure **1** is similar to that of dahlia inulin[9] (having a d.p. of 38).

## EXPERIMENTAL

*Materials and methods.* — Paper partition chromatography (hereafter, "chromatography") was conducted on Whatman No. 1 and 3 MM papers by the descending technique, using the following solvent mixtures (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, and (*B*) 4:1:5 1-butanol–ethanol–water. The staining reagents used were (*a*) alkaline silver nitrate[10], (*b*) aniline hydrogenoxalate[11], (*c*) benzidine periodate[12], and (*d*) alkaline triphenyltetrazolium chloride[10].

All specific rotations were measured, at equilibrium, with a Perkin–Elmer Polarimeter, model 241. Evaporations were performed at 35–40° under diminished pressure. Unless otherwise stated, decationization was performed with Amberlite IR-120 ($H^+$) ion-exchange resin.

G.l.c. was performed with stainless-steel columns containing (I) 3% of SE-52 on Chromosorb WAW (1.83 m × 3.2 mm), (II) 15% of diethylene glycol succinate on Chromosorb WAW (1.83 m × 6.4 mm), and (III) 15% of 1,4-butanediol succinate polyester on Celite (1.83 m × 3.2 mm). A Hewlett–Packard Gas Chromatograph, model 5730A, equipped with a flame ionization detector was used, nitrogen being the carrier gas. Per(trimethylsilyl) derivatives of the sugars were prepared by the

method of Sweeley *et al.*[13]. Partially methylated fructose (~2 mg) was converted into the methyl glycosides by treatment with dry, methanolic hydrogen chloride (0.5%) for 72 h at 28–30°. After removal of the solvent, the residue was dissolved in water (~5 mL), and the acid neutralized with silver carbonate. The suspension was filtered, and the filtrate was de-cationized. The product was evaporated to dryness, and dissolved in chloroform, and aliquots of the solution were subjected to g.l.c.

*beta*-Amylase was obtained from E. Merck, Germany (specific activity 28 units/mg), and *alpha*-amylase from Hanku Kyoei Bussan Co., Japan (specific activity 5 units/mg). β-D-Fructofuranosidase (yeast invertase) was a gift from Sigma, U.S.A. (Grade X, specific activity 600 units/mg).

Total hexose, and fructose, were estimated by the method of Dische *et al.*[14,15]. Spectrophotometric data were recorded with Pye Unicam, model SP 500, and Perkin–Elmer, model 337, spectrophotometers. Methoxyl content was determined by the Zeisel method[16], and high-voltage electrophoresis was conducted with a Shandon apparatus, model L-24.

*Isolation of the fructan component ($B_9$) from polysaccharide $B_2$.*—Polysaccharide $B_2$ (10 g) was extracted with 3:7 (v/v) water–ethanol (4 × 250 mL) by stirring for 1.5 h at room temperature. The combined extracts were clarified by centrifugation at 5,500 r.p.m. for 40 min at 15°. The precipitate was discarded, and the clear, supernatant liquor was concentrated to ~25 mL. The concentrate was cooled to 5°, and treated with cold ethanol (4 vol.), with stirring. The precipitate (7.1 g) was reprecipitated four times with dry ethanol (4 vol.) from its aqueous solution (2%), to afford a white, amorphous, extremely hygroscopic material (polysaccharide $B_9$) having $[\alpha]_{589.5}$ −42.3° (*c* 1.0, water); yield 3.6 g.

*Electrophoresis of polysaccharide $B_9$.* — Polysaccharide $B_9$ (100 μg) was subjected to high-voltage electrophoresis on paper (Whatman No. 1) for 50 min at 4°, using a borate buffer (0.01M, pH 9.85), at a potential gradient of 47V/cm with an average current strength of 24 mA. On development with reagent *C* (with the modification that the paper was kept for 3 min at 60° after being passed through the periodic acid solution, and then dipped into the benzidine reagent), $B_9$ was resolved into two overlapping zones — a blue zone (deeper than the background) and a white zone, having respective mobilities of 4.7 and 2.5 cm towards the cathode. On the same paper, D-glucose moved 10.8 cm towards the anode.

The blue (polysaccharide $B_{10}$) and the white (polysaccharide $B_{11}$) zones were separated by preparative electrophoresis of $B_9$ (200 mg, applied in 30-mg lots on each 12.5-cm wide Whatman No. 3 MM paper) under the same conditions, and separately eluted with water. The eluates were acidified (pH 4.0) with acetic acid (6M) at 4°, kept for 1 h at room temperature, dialyzed, and lyophilized, to yield polysaccharide $B_{10}$ (47.2 mg) and polysaccharide $B_{11}$ (86.2 mg), having the properties given in Table I. On re-electrophoresis of $B_{10}$ and $B_{11}$, their order of mobility remained the same, and their boundaries merged.

*Attempted fractionation of $B_9$ with barium hydroxide.* — To a solution of $B_9$

(88.6 mg) in water (0.9 mL) was added, dropwise, a saturated, aqueous solution (2.0 mL) of barium hydroxide. The resulting precipitate was collected by centrifugation at 4°, washed with several changes of ice-cold water, suspended in water (2.0 mL), and decomposed with carbon dioxide. The precipitate was centrifuged off, and the solution was decationized and lyophilized, to yield polysaccharide $B_{12}$ (40.3 mg) having the properties given in Table I.

*Treatment of polysaccharide $B_9$ with* alpha- *and* beta-*amylase.* — To a solution of $B_9$ (~15 mg) in acetate buffer (0.2M; 3.75 mL, pH 4.8) was added 0.25 mL of a solution (1 mg/mL in the same buffer) of *beta*-amylase, and the mixture was incubated for 24 h at 37°. Similar experiments were set up simultaneously with amylose solution (~15 mg in 3.75 mL of buffer), and a solution containing a mixture of amylose (~5 mg) and $B_9$ (~10 mg) in the same buffer (3.75 mL). A micro-drop from each solution was tested, at intervals, with iodine solution (0.01M). At practically zero time, both of the solutions containing amylose gave the usual blue color, which gradually faded with time, and finally disappeared within 30 min. The solution of polysaccharide $B_9$ did not give a stain with the iodine solution. After 24 h, the tubes of reaction mixtures were immersed for 10 min in a water-bath at 80°, and then centrifuged. The solutions were separately de-ionized, concentrated, and chromatographed (solvent *A*). Only amylose-containing solutions released maltose, along with some oligosaccharides; $B_9$ did not release any reducing sugar.

The experiment was repeated with *alpha*-amylase, and the subsequent observations were the same.

*Treatment of polysaccharide $B_9$ with β-D-fructofuranosidase* (yeast invertase). — To a 1% solution of $B_9$ (2.0 mL) in acetate buffer (0.1M, pH 4.5) was added 0.1 mL of a solution of yeast invertase (1 mg/mL), and the mixture was incubated for 24 h at 55°. Sucrose solution (1%; 2.0 mL) and a mixture of sucrose (1%; 1.0 mL) with $B_9$ (1%; 1.0 mL) were used as control substrates. The progress of the reaction was monitored by periodically testing a micro-drop from each solution with staining reagent *a*. The solutions containing sucrose released reducing sugars within 5 min of addition of the enzyme. After 6 and 24 h, chromatography (solvent *A*; staining reagents *a*, *c*, and *d*) of aliquots (10 μL) from each test sample revealed complete hydrolysis of sucrose within 6 h, both individually and in admixture with $B_9$. Polysaccharide $B_9$ released only fructose at a very low rate, as detected at the sixth hour, and this rate did not increase appreciably, even after 24 h.

*Acetylation and fractionation of polysaccharide $B_9$.* — $B_9$ (2.0 g) was dispersed in *N*,*N*-dimethylformamide (50 mL), and acetylated with acetic anhydride (30 mL) and pyridine (40 mL) by stirring for 2 days at room temperature. The mixture was cooled, diluted with glacial acetic acid (65 mL), and poured into ice-cold water (500 mL) with stirring. The resulting precipitate was filtered off, washed with water until free from acid, drained well, and dried *in vacuo* over $P_2O_5$; yield 2.1 g. This material in pyridine (40 mL) was reacetylated with acetic anhydride (30 mL), and the product (1.9 g) was extracted with methanol (3 × 30 mL), whereby a portion of it dissolved. The residue was subjected to a third acetylation with the same amounts

of the reagents, and the final acetylation product, after isolation in the usual way, was again extracted with methanol (2 × 30 mL). The residue (1.41 g), designated polysaccharide $B_{14}$, had $[\alpha]_{589.3}$ −28.3° (*c* 0.99, chloroform) and was devoid of an absorption band in the hydroxyl region of its i.r. spectrum. The methanol extracts were pooled, and evaporated to dryness under diminished pressure, to afford polysaccharide $B_{13}$, which had $[\alpha]_{589.5}$ −28.7° (*c* 1.0, chloroform). Both $B_{13}$ and $B_{14}$ could be solubilized by rubbing with 70% $H_2SO_4$ at 4°; the solutions were diluted to 0.05M, and hydrolyzed by heating for 4 h on a boiling-water bath. G.l.c. of the hydrolyzate as the per(trimethylsilyl) derivatives gave peaks for glucose and fructose in the ratios 1 : 11.2 for $B_{13}$ and 1 : 13.8 for $B_{14}$. Under similar conditions of hydrolysis, polysaccharide $B_9$ gave glucose and fructose peaks in the ratio of 1 : 13.6.

*Methylation analysis of polysaccharide $B_{14}$.* — Polysaccharide $B_{14}$ (1.4 g) was methylated with dimethyl sulfate and solid sodium hydroxide in tetrahydrofuran, and the partially methylated product (674 mg) was further methylated (six times) by the Purdie method[5], to yield the permethylated polysaccharide (546 mg) in the form of a brown, semi-solid mass having $[\alpha]_{589.5}$ −44.2° (*c* 1.0, chloroform) and OMe 43.6%; there was no OH band in the region of 3600–3200 $cm^{-1}$ in its i.r. spectrum. The product (540 mg) was fractionally precipitated from its chloroform solution (2.0 mL) by adding increasing portions of petroleum ether (b.p. 40–60°). At each stage of turbidity, the precipitate was collected by centrifugation, and re-precipitated from the same solvent-system. Only two fractions were obtained; the first ($b_1$) was a dark-colored, sticky material, and the second ($b_2$) was obtained as orange flakes. The fractionation is summarized in Table III.

*Hydrolysis of $b_2$ and identification of the methylated sugars.* — The methylated fraction $b_2$ (~20 mg) was methanolyzed by heating under reflux for 16 h with dry, methanolic hydrogen chloride (0.25%; 5 mL). After removal of the solvent, the residue was dissolved in water (10 mL), the solution made neutral with $Ag_2CO_3$, and the suspension filtered. The filtrate was decationized, and evaporated to a syrup, $h_1$ (14.1 mg), a mixture of methyl glycosides. This was subjected to g.l.c., using column II (190°) and column III (175°). The results are given in Table IV. Demethylation[7] of $h_1$ (~5 mg) with hydrobromic acid, and chromatography (solvent *A*) of the product revealed the presence of glucose only (along with some tailings).

TABLE III

FRACTIONAL PRECIPITATION OF PERMETHYLATED $B_{14}$

| *Fraction No.* | *Petroleum ether added (mL)* | *Ratio of chloroform to petroleum ether (v/v)* | *Yield (mg)* | $[\alpha]_{589.5}$ *(degrees)*[a] | *OMe (%)* |
|---|---|---|---|---|---|
| $b_1$ | 20 | 1:10 | 109.0 | −41.6 | 42.5 |
| $b_2$ | 40 | 1:20 | 228.3 | −46.4 | 44.1 |

[a]In chloroform.

TABLE IV

RESULTS OF G.L.C. OF HYDROLYZATES $h_1$ AND $h_2$, AND FRACTION $f_1$

| *Description of sample* | *RRT[a] values of the peaks* | | *Sugars detected* |
|---|---|---|---|
| | *Col. II, 190°* | *Col. III, 175°* | |
| Hydrolyzate $h_1$ | (a) 1.05(w), 1.30(sh), 1.46(w) | (a) 1.04(w), 1.28(sh), 1.45(w) | (a) methyl 1,3,4,6-tetra-*O*-methylfructosides and methyl 2,3,4,6-tetra-*O*-methylglucosides |
| | (b) 3.00(s), 4.55(m) | (b) 2.67(s), 4.05(m) | (b) methyl 3,4,6-tri-*O*-methylfructosides |
| | (c) 3.64(sh), 5.35(w) | (c) 3.27(sh), 4.82(w) | (c) methyl 2,4,6-tri-*O*-methylglucosides |
| | (d) 2.17(m), 3.43(sh), 6.68(m) | (d) 1.93(m), 3.06(sh), 6.03(m) | (d) unidentified |
| Hydrolyzate $h_2$ (as methyl glycosides) | (a) 1.06(w), 1.28(sh), 1.46(w) | (a) 1.05(w), 1.27(sh), 1.46(w) | (a) } |
| | (b) 3.00(s), 4.54(m) | (b) 2.68(s), 4.06(m) | (b) } as for $h_1$ |
| | (c) 3.68(sh), 5.41(w) | (c) 3.28(sh), 4.84(w) | (c) } |
| | (d) 2.32(w), 3.44(sh), 6.68(w) | (d) 2.06(w), 3.07(sh), 6.04(w) | (d) } |
| Fraction $f_1$ (as methyl glycosides) | (a) nil | (a) nil | (a) nil |
| | (b) 3.07(s), 4.59(m) | (b) 2.73(s), 4.09(m) | (b) methyl 3,4,6-tri-*O*-methylfructosides |
| | (c) 3.68(sh), 5.36(w) | (c) 3.28(sh), 4.84(w) | (c) methyl 2,4,6-tri-*O*-methylglucosides |
| | (d) 2.36(w), 3.50(sh), 6.77(w) | (d) 2.10(w), 3.12(sh), 6.03(w) | (d) unidentified |

[a]RRT values refer to relative retention times with respect to that of methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside as unity; m = medium, s = strong, sh = shoulder, and w = weak g.l.c. peaks.

TABLE V

RELATIVE RETENTION TIMES (RRT) OF METHYL ETHERS OF D-GLUCOSE AND D-FRUCTOSE

| *Methyl glycosides of* | *RRT values of the peaks* | |
|---|---|---|
| | *Col. II, 190°* | *Col. III, 175°* |
| 2,3,4,6-Tetra-*O*-methyl-D-glucose | 1.00(m), 1.45(s) | 1.00(m), 1.43(s) |
| 2,3,6-Tri-*O*-methyl-D-glucose | 3.82(m), 5.25(s) | 3.56(m), 4.80(s) |
| 2,4,6-Tri-*O*-methyl-D-glucose | 3.71(m), 5.39(s) | 3.31(m), 4.88(s) |
| 2,3,4-Tri-*O*-methyl-D-glucose | 2.91(m), 4.14(s) | 2.56(m), 3.66(s) |
| 1,3,4,6-Tetra-*O*-methyl-D-fructose[a] | | 1.04(s), 1.26(m) |
| 3,4,6-Tri-*O*-methyl-D-fructose[a] | | 2.74(s), 4.12(m) |
| 1,3,4-Tri-*O*-methyl-D-fructose[a] | | 1.89(w), 2.49(s), 3.94(m), 4.43(m) |

[a]Lit.[17] values.

Another portion (~5 mg) of $h_1$ was hydrolyzed with aqueous oxalic acid (1%; 1 mL). The hydrolyzate was made neutral with $CaCO_3$, and filtered; the filtrate was decationized, and evaporated to a syrup. On chromatography (solvent *B* and staining reagent *b*), this furnished a brownish-yellow, elongated spot ($R_G$* 0.93) corresponding to 2,4,6-tri-*O*-methylglucose, and a pink spot ($R_G$ 1.00) corresponding to 2,3,4,6-tetra-*O*-methylglucose. On developing another part of the same paper with staining reagent *d*, a single red spot ($R_G$ 0.86) appeared.

In a separate experiment, the methylated fraction $b_2$ (~30 mg) was hydrolyzed with 1% oxalic acid in 70% (v/v) aqueous methanol (6 mL) by heating under reflux for 18 h, and the solution was processed as before. In chromatography (solvent *A* and staining reagents *b* and *d*), the hydrolyzate, $h_2$ (24.3 mg), furnished results similar to the preceding. The portion of $h_2$ that gave a red stain with reagent *d* was isolated by preparative chromatography. This fraction, $f_1$ (9.6 mg), had $[\alpha]_{589.5}$ +32.5° (*c* 0.96, water) (lit.[6] +30.3°) and OMe 40.2%. Portions of $h_2$ and of $f_1$ (~2 mg each) were converted into their methyl glycosides, and these were subjected to g.l.c. using column II (190°) and column III (175°). The results are given in Table IV. Table V gives RRT values found for authentic samples (or literature values) of some methyl ethers of D-glucose and D-fructose.

Another portion (~2 mg) of $f_1$ was treated with aqueous sodium hydroxide (0.1M, 1 mL) for 48 h at room temperature under nitrogen. The solution was decationized with Dowex-50W X-8 ($H^+$) resin, and evaporated to a syrup. After demethylation[7], chromatography (solvent *A* and staining reagents *a* and *d*) of the syrup revealed the presence of glucose and mannose and/or fructose. On treatment with hydrobromic acid under identical conditions and chromatography (same solvent and staining reagents), D-fructose (~2 mg) did not furnish any spot corresponding to the position of fructose and/or mannose.

*$R_G$ values were determined with respect to 2,3,4,6-tetra-*O*-methyl-D-glucose as unity.

*Periodate oxidation of $B_9$ and $B_{11}$.* — Polysaccharides $B_9$ (4.32 mg; in duplicate) and $B_{11}$ (3.91 mg; also in duplicate) were separately oxidized, in the absence of light, with sodium metaperiodate solution (0.04M) at 4°, and the uptake of periodate was determined at intervals by the spectrophotometric procedure[18]. The uptake became practically constant in 12 h, when $B_9$ and $B_{11}$ had reduced 0.96 and 0.95 mole, respectively, of the oxidant per hexosyl residue.

In another experiment, $B_9$ (100 mg, in 50 mL of water; in duplicate) was oxidized with sodium metaperiodate solution (0.04M, 50 mL) in the dark at 4°. Aliquots (25 mL) were withdrawn from the duplicate reaction-mixtures after 24, 48, and 72 h, and the formic acid liberated was estimated with 0.01M sodium hydroxide solution. One molar equivalent of formic acid was liberated by the oxidation of 51.05 moles (average value) of hexosyl residues. After determination of the formic acid, the duplicate oxidation-mixtures were pooled, and the excess of periodate was decomposed with ethylene glycol (2.0 mL). After extensive dialysis at 4°, and lyophilization of the solution to 10 mL, the oxidized polysaccharide was reduced at 10° with potassium borohydride (500 mg). The excess of borohydride was decomposed with ice-cold acetic acid (6M), and the solution was dialyzed and lyophilized, to afford the reduced polysaccharide (9.6 mg). Chromatography (solvent *A* and staining reagents *a*, *c*, and *d*) of the hydrolyzate of a portion (~2 mg) of the product revealed the presence of glycerol (major component) and a little glucose, and traces of some unidentified spots moving faster than glucose; there was no fructose. Estimation[14] (by measurement of the absorbance at 415 nm) showed that 1.83% of hexose survived the oxidation. The oxidation and reduction procedure was repeated once more on the previously oxidized and then reduced $B_9$. The results of chromatography (solvent *A* and staining reagents *a* and *c*) of the hydrolyzate of the resulting material (5.6 mg) corroborated the earlier findings. The proportion of periodate-resistant hexose was estimated to be 1.74% in this case.

Similar Smith-degradation was performed on $B_{11}$ (30.6 mg), and the resulting degraded product (5.2 mg) also contained glucose (1.79%).

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REFERENCES

1 N. N. Das, A. Das, and A. K. Mukherjee, *Carbohydr. Res.*, 56 (1977) 337–349.
2 H. Belval, *Bull. Soc. Chim. Biol.*, 25 (1943) 64–67.
3 H. Hibbert, R. S. Tipson, and F. Brauns, *Can. J. Res.*, 4 (1931) 221–239.
4 J. K. Hamilton and H. W. Kircher, *J. Am. Chem. Soc.*, 80 (1958) 4703–4709.
5 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 83 (1903) 1021; 85 (1904) 1049–1070.
6 D. J. Bell and A. Palmer, *J. Chem. Soc.*, (1952) 3763–3770.
7 L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.*, (1950) 1702–1706.

8 D. J. BELL AND R. DEDONDER, *J. Chem. Soc.*, (1954) 2866–2870.
9 E. L. HIRST, D. I. MCGILVRAY, AND E. G. V. PERCIVAL, *J. Chem. Soc.*, (1950) 1297–1302.
10 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444–445.
11 R. H. HORROCKS AND G. B. MANNING, *Lancet*, 256 (1949) 1042–1045.
12 J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132–1134.
13 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.
14 Z. DISCHE, L. B. SHETTLES, AND M. OSNOS, *Arch. Biochem.*, 22 (1949) 169–184.
15 Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 192 (1951) 583–587.
16 E. P. CLARK, *Semimicro Quantitative Organic Analysis*, Academic Press, New York, 1943, pp. 68–72.
17 C. T. BISHOP, *Adv. Carbohydr. Chem.*, 19 (1964) 112–113.
18 J. S. DIXON AND D. LIPKIN, *Anal. Chem.*, 26 (1954) 1992–1993.