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SUGARS FROM *Sphacelia sorghi* HONEYDEW

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ABSTRACT

Several unusual oligosaccharides have been isolated from the honeydew of *Sphacelia sorghi* McRae. These include 1-*O*-*β*-D-fructofuranosyl-D-mannitol, 5-*O*-*β*-D-fructofuranosyl-D-arabinitol, 1,6-di-*O*-*β*-D-fructofuranosyl-D-mannitol, 1,5-di-*O*-*β*-D-fructofuranosyl-D-arabinitol, and 1-*O*-*β*-D-fructofuranosyl-6-*O*-[*β*-D-fructofuranosyl-(2→6)-*β*-D-fructofuranosyl]-D-mannitol. In addition to these oligosaccharides, D-glucose, D-fructose, D-arabinitol, D-mannitol, sucrose, and 6-*O*-*β*-D-fructofuranosyl-D-glucose were also found in the honeydew. The structures of the previously undescribed oligosaccharides were determined by periodate oxidation studies, their cleavage by *β*-D-fructofuranosidase, optical rotation measurements, and methylation analysis by combined gas–liquid chromatography–mass spectrometry. The position of linkage in the arabinitol-containing disaccharide was determined by incorporation of D-[1-^{3}H]-arabinitol into a *β*-D-fructofuranosyl-D-arabinitol *in vivo*. The release of tritium-labeled formaldehyde during periodate oxidation of the product demonstrated that the *β*-D-fructofuranosyl moiety was linked to position 5 of the D-[1-^{3}H]-arabinitol.

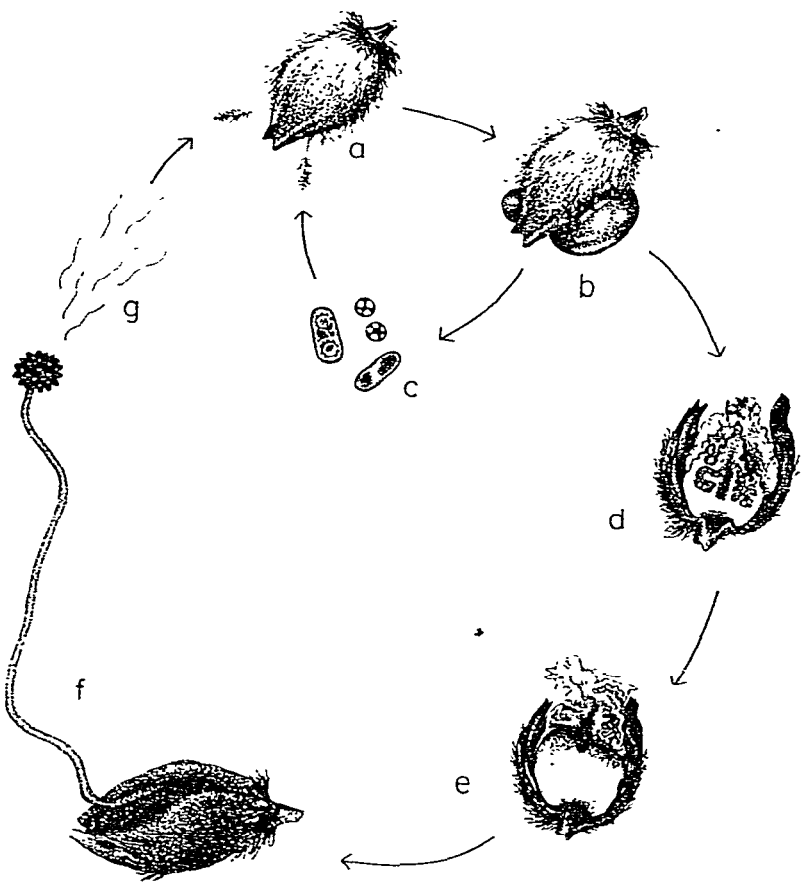
INTRODUCTION

Sphacelia sorghi is an imperfect (asexual) parsitic fungus which infects the ovaries of *Sorghum*. Shortly after infection the floral cavity becomes filled with the fungal sphacelium, a spore-bearing structure that exudes a sugary exudate called honeydew. Sphacelial honeydews contain great numbers of conidia (fungal spores), which distinguish them from insect honeydews. When *Sphacelia* honeydews are carried to other flowers, the conidia initiate new infections. Insects and rain are the major means of transmission, and during wet years *S. sorghi* may reach epidemic proportions. The sphacelial stage of the sorghum fungus later becomes the sclerotium, or ergot, a hard creamy-white resting structure which takes the place of the cereal's seed.

In all of the other important ergot diseases of cereals, the ergot subsequently develops the sexual spores (ascospores) of the fungus, the perfect stage which is known as *Claviceps*. In the sorghum ergot, however, the ascospore stage has not hitherto been described, even though large crop losses may result from the activities of this fungus in the tropics and subtropics. Hence, the ability of *S. sorghi* to reappear

following a long dry season has remained a mystery. Recently, Mower (unpublished) has discovered the *Claviceps* sexual stage of *S. sorghi*, developing from the sclerotia which remain viable during dry periods and germinate under the wet conditions of the next rainy season. As in other species, the long, filamentous ascopores are liberated into the air and are carried about by the wind to start, presumably, the new infections. The life cycle of the sorghum ergot is illustrated in Scheme I.

All *Claviceps* species produce sphacelial honeydew. The honeydew is important both from the standpoint of epidemiology and carbon assimilation[1]. Epidemiologically, the honeydew sugars function in preservation of the conidia during dry periods



Scheme. I. Life cycle of the sorghum ergot. Illustrations were drawn at the indicated magnifications: (*a*) detached flower of sorghum (× 5) showing the two stigmas as sites of infection; (*b*) honeydew (× 5) exuding from the detached flower nine days after inoculation; (*c*) enlargement of honeydew showing the conidia (× 500) which are embedded in the sugary matrix; (*d*) longitudinal section through illustration (*b*) showing sphacelium; (*e*) longitudinal section of later stage which has rounded sclerotium at the base of the floral cavity and the displaced senescent sphacelium; (*f*) ascogenous fruit-body (× 6.5) originating from sclerotium; (*g*) ascospores (× 50) ejected from perithecia in spiny capped fruit-body (drawings by L. Edwards).

and in attraction of insect vectors for dispersal of conidia to other flowers. As to carbon assimilation, it has been suggested[1] that the breakdown of host sucrose is brought about by the parasite's wall-bound β-D-fructofuranosidases and β-D-trans-fructofuranosidases. The enzymes presumably remain localized in the floral cavity because the fungal hyphae never penetrate beyond the host region delimited by the seed (compare Scheme I). Hence the floral cavity becomes a zone of low sucrose concentration, and a continual net flow of this sugar to the infection site results. In most species of ergot, several sugars are formed in the honeydew, and the resulting higher osmotic concentration in the floral cavity serves to draw in host fluids.

Because of the particular host–parasite relations of *Claviceps*, as well as its economic importance, characterization of the sphacelial sugars has been a matter of special interest. The sugars of *C. purpurea*, ergot of rye, were partially described in earlier reports[2,3] and, more recently, four oligosaccharides were reported in that species[4]. A number of investigators have characterized carbohydrate components in sucrose broth cultures of the mycelium of *Claviceps* spp.[4–6], but the carbohydrates of the sorghum fungus have not been reported previously. The present paper deals mainly with the description of five new oligosaccharides from *Sphacelia sorghi* McRae.

RESULTS

Lyophilized honeydew, free of spores and pollen (see Experimental, *Honeydew*), was fractionated on a column of Bio-Gel P-2 (Fig. 1). Twenty different sugar species from the fractionated honeydew were resolved by paper chromatography in solvents A and B. All but one of the twelve major components (Fig. 2) were characterized. Of these, six sugars occurring in column peaks 1, 2, and 3 from the Bio-Gel P-2 were of known structure: peaks 4, 5, and 6 contained unknown oligosaccharides that subsequently were characterized.

The monosaccharide fraction (peak 1 in Fig. 1) was composed mainly of glucose and fructose (Fig. 2), the identities of which were confirmed by paper chromatography and enzymic analysis. The disaccharide fraction (peak 3 in Fig. 1) contained 6-*O*-β-D-fructofuranosyl-D-glucose[7], sucrose, D-mannitol, and D-arabinitol. Monitoring of the refractive index of the effluent from the Bio-Gel P-2 column revealed an additional peak (peak 2), which contained D-mannitol and D-arabinitol in a ratio of 65:35, as determined by g.l.c. of the acetylated mixture.

The components of peaks 4, 5, and 6 were purified by chromatography in solvents A and C on Whatman No. 3MM paper sheets. Each strip, containing the resolved sugar, was cut from the chromatogram and eluted with water. Each fraction was passed through a Sephadex G-25 column to separate the sugar from impurities derived from the paper strip. Peak 4 contained disaccharides that were resolved from those in peak 3 because, as part of their structure, they contained an alditol, which increased the effective size by about one hexose residue[8]. One of the major sugars in peak 6 was characterized as a trifructosylmannitol, but the other major component was only partially characterized.

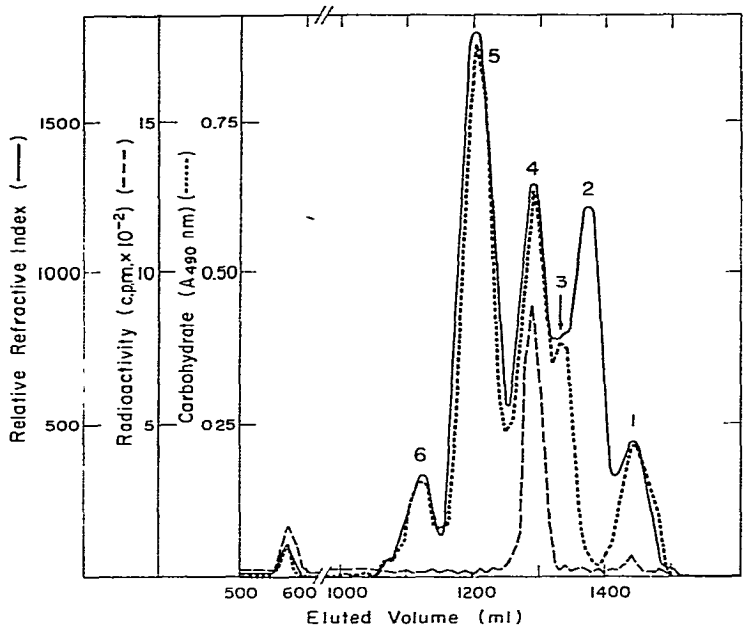


Fig. 1. Honeydew fractionated on a Bio-Gel P-2 column (200–400 mesh) was eluted with water saturated with chloroform. The void volume appeared at 575 ml. The dashed line represents 6-*O*-*β*-D-fructofuranosyl-D-[1-^{3}H]glucitol, which was added as an internal standard. Total hexose was measured by the phenol–sulfuric method (dotted line). Changes in the refractive index (solid line) were plotted to indicate the content of both hexoses and alditols through arbitrary units.

Mannitol-containing disaccharide. — Compound **4a** was cleanly separated from sucrose and had an elution volume on Bio-Gel P-2 identical with that of 6-*O*-*β*-D-fructofuranosyl-D-[1-^{3}H]glucitol (Fig. 1). Fructosylalditols have smaller elution volumes than sucrose on molecular sieves because of the greater apparent size of the linear alditol moiety[8].

Complete acid hydrolysis of **4a** yielded mannitol and fructose, whereas partial acid hydrolysis yielded mannitol, fructose, and **4a**, with no evidence for any product of intermediate molecular size. Digestion of **4a** with invertase yielded mannitol and fructose, which were separated on a Bio-Gel P-2 column, and the mannitol was crystallized from 90% ethanol as needles. The mannitol from **4a** had $[\alpha]_{546}^{25}$ +37.8° (*c* 2.05, in 6.4% borate), and authentic D-mannitol had $[\alpha]_{546}^{25}$ +38.6° (*c* 2.28, in 6.4% borate). Since the disaccharide had a negative rotation (Table I) and was cleaved by invertase, the fructose must have had the *β*-D-furanose configuration.

Periodate oxidation of **4a** generated 2.5 moles of formic acid and 1.23 moles of formaldehyde per mole of disaccharide, and 4.98 moles of periodate were consumed. The consumption of 5 moles of periodate would be consistent with a disaccharide containing a fructofuranose residue linked at position 1 of mannitol. Although 3 moles of formic acid would be expected from 1-*O*-*β*-D-fructofuranosyl-D-mannitol, the

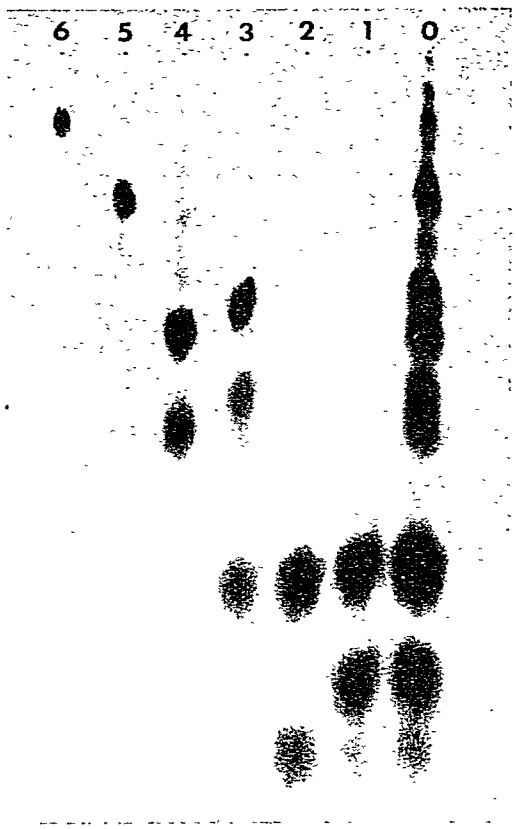


Fig. 2. Components of Bio-Gel P-2 column fractions were separated by solvent A on Whatman No. 1 paper. Chromatograms were developed for 48 h at 25° and then stained with alkaline silver reagent. Origins 1–6 were spotted with aliquots from each of the corresponding peaks in Fig. 1. Origin 0 was the unfractionated honeydew. Each spot is described in descending order. The material from peak 1 was mainly glucose and fructose with a small amount of arabinitol. Components of peak 2 are mannitol and arabinitol. Peak 3 is composed mainly of 6-*O*-β-D-fructofuranosyl-D-glucose, sucrose, and mannitol with a small amount of arabinitol. The composition of peak 4 was comprised of small amounts of several undetermined sugars and large amounts of **4a** and **4b**. Peak 5 was almost entirely **5a**; a minor component **5b** ran slightly faster and was barely detected. The one spot seen in 6 was comprised mainly of **6a** and **6b** and a small amount of **6c**. These tetrasaccharides were resolved after 150 h in solvent A, but poor resolution was obtained upon trying to isolate **6a** and **6b** on Whatman No. 3MM paper.

results obtained with known compounds demonstrated that the somewhat low value obtained was characteristic of the enzymic method employed (Table I).

The acetates of the partially methylated alditols derived from **4a** by methylation, hydrolysis, reduction, and acetylation, were analyzed by g.l.c.-mass spectrometry[9]. Two major g.l.c. peaks were observed in nearly equal proportions. Peak 1 had an R_T value identical with that of 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylmannitol and gave an identical mass spectrum (Table II). Peak 2 had the R_T value of a 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol (see Experimental, *Methylated alditol acetate*)

TABLE I

PROPERTIES AND COMPOSITIONS OF OLIGOSACCHARIDES FROM HONEYDEW

Compound	$[\alpha]_{546}^{25}$	*Products of acid or invertase hydrolysis*[a]	*Periodate consumed*[b]	*Formate produced*[b]	*Formaldehyde produced*[b]
4a	−34.5°	Man(1), Fru(1)	4.98 (5)	2.50 (3)	1.23 (1)
4b	−34.5°	Man(1), Fru(1)	4.15 (4)	1.77 (2)	1.06 (1)
5a	−43.8°	Man(1), Fru(2)	5.03 (5)	1.88 (2)	0.24 (0)
5b	−43.3°	Man(1), Fru(2)	3.94 (4)	0.95 (1)	0.21 (0)
6a	−43.8°	Man(1), Fru(3)	5.70 (6)	1.97 (2)	0.11 (0)
6b	−41.0°	Man(1), Fru(3)	5.37	1.83	0.09
Sucrose	+73.9°	Glc(1), Fru(1)	2.91 (3)	0.83 (1)	0.08 (0)

[a]Molar proportions are given in parentheses. [b]Calculated values in parentheses are for the structures in Fig. 3.

and gave an identical mass spectrum. These data are consistent with the structure 1-*O*-*β*-D-fructofuranosyl-D-mannitol (**4a**) shown in Fig. 3.

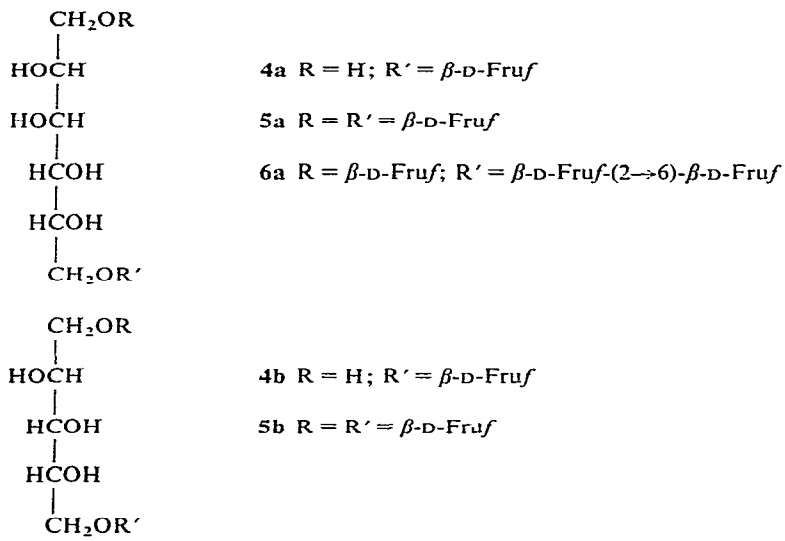


Fig. 3. Proposed structures of oligosaccharides from *S. sorghi* honeydew.

Arabinitol-containing disaccharide. — A second disaccharide (**4b**), was also isolated from peak 4 (Fig. 2), and its elution volume was slightly larger than that of **4a**, indicating that its molecule was slightly smaller. Hydrolysis of **4b** with invertase yielded D-fructose and arabinitol, as identified by paper chromatography in solvents A and B. No sugar of intermediate size between **4b** and the monosaccharides was observed after partial acid hydrolysis. The products of complete acid hydrolysis were separated on a column of Bio-Gel P-2, the column effluent being monitored by

TABLE II

PRIMARY FRAGMENTS IN THE MASS SPECTRA OF PARTIALLY METHYLATED ALDITOL ACETATES SEPARATED BY G.L.C.

Alditol derivatives	R_T	*m/e*								
		43	*45*	*89*	*117*	*161*	*177*	*189*	*205*	*249*
1-*O*-Acetyl-2,3,4,5,6-penta-*O*-methylmannitol	1.00	+	+	+	+	+	+			+
2,5-Di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol	1.62	+	+			+			+	
1,6-Di-*O*-acetyl-2,3,4,5-tetra-*O*-methylmannitol	2.49	+			+	+				
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol	3.68	+	+			+		+		
2,5,6-Tri-*O*-acetyl-1,3,4-tri-*O*-methylhexitol	3.68	+	+			+		+		
Products derived from unknown oligosaccharides										
4a	1.00	+	+	+	+	+	+			+
	1.62	+	+			+			+	
4b	0.51	+	+	+	+	+				
	1.62	+	+			+			+	
5a	1.62	+	+			+			+	
	2.49	+			+	+				
5b	1.45	+			+	+				
	1.62	+	+			+			+	
6a	1.62	+	+			+			+	
	2.49	+			+	+				
	3.68	+	+			+		+		
6b	1.62	+	+			+			+	
	2.49	+			+	+				
	3.68	+	+			+		+		

continual measurement of the refractive index. Fructose and arabinitol appeared as two separate peaks of equal intensity, indicating that **4b** contained equimolar amounts of the two components. The arabinitol fraction was acetylated and the product crystallized as prismatic leaves from 1:5 ethanol–heptane. The m.p. of a mixture of arabinitol pentaacetate from **4b** and authentic D-arabinitol pentaacetate was 72.5–74.3°. A mixture of arabinitol pentaacetate from **4b** and authentic L-arabinitol pentaacetate melted incompletely around 73°, and then resolidified and melted at 93.2–95.3°, a value characteristic of the racemate. The $[\alpha]_{546}^{25}$ of authentic D-arabinitol pentaacetate (*c* 0.43, ethanol) was +49.8°, whereas arabinitol pentaacetate from **4b** gave +49.3° (*c* 0.58, ethanol). Thus, the arabinitol from **4b** had the D-configuration, and the negative rotation of **4b** (Table I) and its cleavage by invertase indicate that the fructose residue had the *β*-D-furanose configuration.

Periodate oxidation of **4b** yielded 1.77 moles of formic acid and 1.06 moles of formaldehyde, and 4.15 moles of periodate were consumed per mole. From these data, the structure of **4b** appeared to be either a 1- or 5-*O*-*β*-D-fructofuranosyl-D-arabinitol. To determine the linkage position, D-[1-^{3}H]-arabinitol was added to a sucrose medium (TS5) containing *S. sorghi*, and the ^{3}H-**4b** produced by the mycelium was isolated and purified as already described. On periodate oxidation of one portion of ^{3}H-**4b**, ^{3}H-CH_2O was liberated in quantitative yield, as assayed by its incorporation into a lutidine derivative (Fig. 4). Another portion of ^{3}H-**4b** was submitted to periodate oxidation and the product was reduced with sodium borohydride and then acidified to pH 5. The reaction mixture was evaporated at 30° to remove the resulting ^{3}H-methanol. After boric acid had been removed as methyl borate, the radioactivity in the residue was measured. The nonvolatile radioactivity corresponded to only 1.5% of that in the starting material. The results of these two experiments demonstrate that the *β*-D-fructofuranose was linked to position 5 of D-arabinitol.

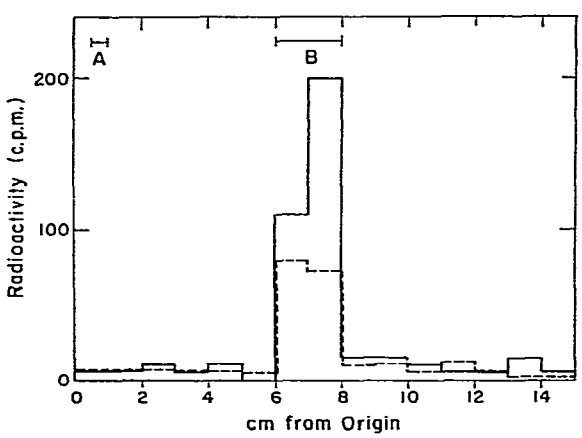


Fig. 4. Thin-layer chromatogram of the radioactive lutidine derivatives obtained from periodate oxidation of [1-^{3}H]-arabinitol (solid line) and of ^{3}H-4b (dashed line). The yellow spots A and B were observed on the plastic-backed thin-layer plates developed in Solvent E. Spot B corresponded to 3,5-diacetyl-1,4-dihydro-2,6-lutidine.

The acetates of the partially methylated alditols, derived from **4b** by the reactions described previously, were analyzed by g.l.c.–mass spectrometry. Peak 1 (5-*O*-acetyl-1,2,3,4-tetra-*O*-methylarabinitol) had an R_T value approximately half that of 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylmannitol, but the prominent peaks in the mass spectra were identical with those of the latter (Table II). Decreased intensities of minor peaks having *m/e* 145 and 177 probably resulted from small differences between the fragmentation patterns of the two compounds. Peak 2 from g.l.c. had an R_T value and mass spectrum identical with those of 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol. These data are consistent with the structure of **4b** shown in Fig. 3, 5-*O*-β-D-fructofuranosyl-D-arabinitol.

Mannitol-containing trisaccharide. — On a Bio-Gel P-2 column, **5a** had the elution volume of a difructosylhexitol. Both acid and enzymic hydrolysis yielded fructose and mannitol in a 2:1 ratio. Partial acid hydrolysis of **5a** yielded D-mannitol, $[\alpha]_{646}^{25}$ +38.4° (*c* 2.25, in 6.4% borate), fructose, and an intermediate product having the properties of **4a**. The cleavage of **5a** by invertase, and its negative rotation (Table I), suggest that both fructose residues had the β-D-furanose configuration. Each mole of **5a** consumed 5.03 moles of periodate. The generation of 1.88 moles of formic acid and only 0.24 moles of formaldehyde suggested that the β-D-fructofuranosyl groups were linked at positions 1 and 6 of the D-mannitol.

The acetates of the partially methylated alditols derived from **5a** were investigated by g.l.c. and mass spectrometry. Peak 1 in the g.l.c. tracing had an R_T value and mass spectrum identical with those of 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol, presumably derived from the two terminal fructofuranosyl residues. Peak 2 contained about one-half the area of peak 1 and had an R_T value and mass spectrum identical with 1,6-di-*O*-acetyl-2,3,4,5-tetra-*O*-methylmannitol. The mass spectrum of peak 2 lacked an *m/e* 45 fragment, denoting the absence of a primary methyl ether. From these data, it was concluded that **5a** was 1,6-di-*O*-β-D-fructofuranosyl-D-mannitol (Fig. 3).

Arabinitol-containing trisaccharide. — On the Bio-Gel P-2 column, **5b** was not resolved from **5a** although it did have a slightly larger elution volume. Complete acid hydrolysis of **5b** yielded a 2:1 ratio of fructose to arabinitol. Arabinitol pentaacetate derived from **5b** had a melting point and optical rotation identical with those of the D-arabinitol pentaacetate obtained from **4b**. Invertase cleaved **5b** into arabinitol and D-fructose. The susceptibility to invertase hydrolysis and the negative rotation of **5b**, (Table I) showed that both fructose residues had the β-D-furanose configuration.

Periodate oxidation of **5b** liberated 0.95 moles of formic acid and 0.21 moles of formaldehyde per mole, with the consumption of 3.94 moles of periodate. Gas chromatography of the partially methylated alditol acetates derived from **5b** revealed two major components. Peak 1 had a mass spectrum almost identical with that of 1,6-di-*O*-acetyl-2,3,4,5-tetra-*O*-methylmannitol, and lacked an *m/e* 45 fragment, evidence that the arabinitol derivative of **5b** was substituted at both primary hydroxyl groups. We conclude that peak 1 was 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylarabinitol. Peak 2, which was approximately twice the size of peak 1, had an R_T value and mass

spectrum identical with that of 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol. Thus, **5b** had the structure 1,5-di-*O*-*β*-D-fructofuranosyl-D-arabinitol (Fig. 3).

Mannitol-containing tetrasaccharide **6a**. — The mannitol-containing tetrasaccharides were isolated in low yields. It appeared that **6a** was a trifructosyl-hexitol from its elution volume on a Bio-Gel P-2 column. Enzymic cleavage by invertase and the negative optical rotation together proved that the fructose moieties of **6a** had the *β*-D-furanose configuration.

Periodate oxidation of **6a** did not release formaldehyde. The consumption of 5.7 moles of periodate and the generation of 1.97 moles of formic acid per mole suggested that **6a** was a fructosyl derivative of **5a**, with the additional *β*-D-fructofuranose residue linked at position 1 or 6 of fructose. The internal fructose residue should be converted into either 1,3,4- or 3,4,6-tri-*O*-methylfructose after methylation and hydrolysis, but these two derivatives become indistinguishable after conversion into their alditol acetates by reduction with borohydride. Thus, both 2,5,6-tri-*O*-acetyl-1,3,4-tri-*O*-methylhexitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol, obtained by borohydride reduction of the corresponding fructose derivatives, gave primary fragments having *m/e* 161 and 189 by fragmentation of the labile C-3 to C-4 bond[9]. However, reduction at C-2 with sodium borodeuteride increases by 1 the *m/e* values of fragments derived from that end of the molecule. In the derivatives prepared by borodeuteride reduction, the primary fragments of *m/e* 162 and 189 are obtained from the 1,3,4-tri-*O*-methyl derivative, whereas primary fragments of *m/e* 161 and 190 would be obtained from the 3,4,6-tri-*O*-methyl derivative. When analyzed in this fashion, **6a** yielded a trimethylhexitol peak on g.l.c. with prominent fragments having *m/e* 162 and 189, thus identifying it as a 2,5,6-tri-*O*-acetyl-2-*C*-deuterio-1,3,4-tri-*O*-methylhexitol. The mannitol tetrasaccharide **6a**, must have the structure of **5a** with a *β*-D-fructofuranosyl moiety attached to position 6 of one of the other two fructose residues (Fig. 3).

Mannitol-containing tetrasaccharide **6b**. — This compound had an elution volume identical with that of **6a** on gel filtration. Preparative paper chromatography in solvents A and B failed to separate **6b** completely from **6a** and a minor component **6c**. Hence the following results were obtained from slightly impure material. Invertase cleaved **6b** into D-fructose and mannitol. Periodate oxidation liberated 1.83 moles of formic acid and 0.09 moles of formaldehyde, with a consumption of 5.37 moles of periodate per mole of **6b**. Although **6b** may, like **6a**, be a derivative of **5a**; the pierodate consumption suggested that the third fructose was bound to position 3 or 4 of one of the fructose residues of **5a**.

However, following methylation of **6b**, g.l.c. revealed a tri-*O*-methylhexitol peak having the same R_T value as that obtained from **6a**. Unfortunately, the mass spectrum showed paired peaks of *m/e* 189+190 and 161+162 from the primary fragments. This would indicate a mixture of 2-*C*-deuterio-3,4,6-tri-*O*-methyl and 2-*C*-deuterio-1,3,4-tri-*O*-methylhexitol acetates. Peaks corresponding to 1,6-di-*O*-acetyl-2,3,4,5-tetra-*O*-methylmannitol and 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol were also present.

Culture-broth oligosaccharides. — To determine whether *S. sorghi* produced the same sugars in parasitic and saprophytic environments, the oligosaccharides found in the honeydew and those produced in liquid cultures were compared. The mycelium grown on sucrose plus alditols produced the same sugars found in the honeydew. Although D-mannitol and D-arabinitol were produced in unsupplemented TS5 medium, their concentrations apparently did not reach a level that would allow production of the high amounts of fructosylalditols found in the honeydew. These oligosaccharides were formed readily in TS5 medium when supplemented with 1% each of D-mannitol and D-arabinitol. The R_F values of these sugars, in solvents A and C, were identical with those of 6-*O*-*β*-D-fructofuranosyl-D-glucose, 5-*O*-*β*-D-fructofuranosyl-D-arabinitol, 1-*O*-*β*-D-fructofuranosyl-D-mannitol, and 1,6-di-*O*-*β*-D-fructofuranosyl-D-mannitol. As in honeydew, the 1,5-di-*O*-*β*-D-fructofuranosyl-D-arabinitol was formed only in small proportion. When comparing stationary and shake cultures, the sequence in which the various oligosaccharides appeared in the medium was the same, but the overall rate of production was somewhat lower in the former.

DISCUSSION

In addition to D-glucose, D-fructose, D-arabinitol, D-mannitol, sucrose, and 6-*O*-*β*-D-fructofuranosyl-D-glucose, we have shown that honeydew from *Sphacelia sorghi* McRae contains 1-*O*-*β*-D-fructofuranosyl-D-mannitol, 5-*O*-*β*-D-fructofuranosyl-D-arabinitol, 1,6-di-*O*-*β*-D-fructofuranosyl-D-mannitol, and 1,5-di-*O*-*β*-D-fructofuranosyl-D-arabinitol, as well as two trifructofuranosylmannitol derivatives. Since most of these compounds were produced by the fungus grown in pure culture, the enzymes of the parasite, and not the host, are responsible for synthesis of the major sugar components in the honeydew of *S. sorghi*. Furthermore, these results and those reported previously[4] support the hypothesis that sucrose is the major carbon source for the ergot fungi.

Although the overall sugar compositions of honeydews of different *Claviceps* species appear to be chromatographically distinct, some sugar components are common to several of them[1]. This implies that a species specificity of the trans-*β*-D-fructofuranosidases is involved in their biosynthesis and/or a preferential reabsorption of certain sugars occurs subsequent to their biosynthesis.

A case can be made for differences in enzyme specificity if one compares the sugars produced by the three different *Claviceps* in Table III. All three species produce 6-*O*-*β*-D-fructofuranosyl-D-glucose. However, *C. species* and *S. sorghi* appear to lack an enzyme to link a D-fructofuranose residue to either the foregoing disaccharide or to position 6 of the D-glucose moiety in sucrose. *C. species* has the ability to link a D-fructofuranosyl residue to position 1 of a *β*-D-fructofuranosyl glycoside, as does *C. purpurea*, whereas *S. sorghi* appears to lack the enzyme for this conversion. The sorghum ergot may have a specific transferase for the D-*erythro* configuration of alditols, as implied from the ratios of **4a** to **5a** and **4b** to **5b** (Figs. 1 and 2). Moreover, the fructose in **4b** is almost entirely linked to the D-*erythro* portion of D-arabinitol.

TABLE III

COMPARISON OF OLIGOSACCHARIDE DISTRIBUTION IN ERGOT

Fungus	*Oligosaccharide**								
	a	b	c	d	e	f	g	h	i
S. sorghi	+	+	+	±	+	−	−	−	−
C. purpurea	−	−	−	−	+	+	+	+	−
C. species†	−	−	−	−	+	+	−	−	+

*a, 1-*O*-*β*-D-Fru*f*-D-mannitol; b, 5-*O*-*β*-D-Fru*f*-D-arabinitol; c, 1,6-di-*O*-*β*-D-Fru*f*-D-mannitol; d, 1,5-di-*O*-*β*-D-Fru*f*-D-arabinitol; e, 6-*O*-*β*-D-Fru*f*-D-Glc; f, *O*-*β*-D-Fru*f*-(2→1)-*O*-*β*-D-Fru*f*-(2→6)-D-Glc; g, *O*-*β*-D-Fru*f*-(2→6)-*O*-*α*-D-Glc*p*-(1→2)-*β*-D-Fru*f*; h, *O*-*β*-D-Fru*f*-(2→1)-*O*-*β*-D-Fru*f*-(2→6)-*O*-*α*-D-Glc*p*-(1→2)-*β*-D-Fru*f*; i, *O*-*β*-D-Fru*f*-(2→1)-*O*-*β*-D-Fru*f*-(2→1)-*O*-*β*-D-Fru*f*-(2→6)-D-Glc.

†Although the *C. species* has been designated *C. purpurea* (Stevens et Hall strain 275) by Arcamone *et al.*[5], there is some doubt as to the validity of their species determination. *C. purpurea* was described by Tulasne, not Stevens and Hall, and a comparison of the oligosaccharide content of the honeydews of several wildtype strains of *C. purpurea* from our laboratory agree with the results of Bassett *et al.*[4]. P. G. Mantle (personal communication) maintains that strain 275 is, in fact, *C. purpurea* (Fr.) Tul.

Alditol hexosides have been reported both in *Claviceps* and other parasitic and mutualistic fungi. Clavicepsin, a diglucoside of mannitol, was reported in the sclerotia of *Claviceps purpurea*[10]. *Ustilago* spp. contain an erythritol mannoside, free or esterified with fatty acids[11]. Umbilicin, an arabinitol galactoside, was originally isolated from the lichen *Umbilicaria pustulata*[12]. Both a mannitol glucoside and a galactoside occur in the thalli of *Peltigera* spp.[13] and *Lichina pygmea* has been reported to contain a small amount of a mannitol mannoside[14].

EXPERIMENTAL

General. — Optical rotations were measured on a Bendix Model 1100 polarimeter, and gel filtration columns were monitored by a Laboratory Data Control Model 1103 RefractoMonitor. Melting points were taken in capillaries on a Thomas "Uni-melt"apparatus. Kinetics, both of hydrolysis and periodate oxidation, were monitored with a Cary Model 14 recording spectrophotometer, and colorimetric assays were read on a Zeiss spectrophotometer. The Park–Johnson method[15] was used to determine the amount of reducing sugar in the Bio-Gel P-2 fractions, and CalBiochem Glucose Stat-Pack was used to determine D-glucose concentrations in the hydrolyzed fractions. Combined g.l.c.–mass spectrometry was performed on a DuPont Model 21-491 instrument at an ionizing potential of 70 eV. A 3 feet by 0.125 inch stainless-steel column, packed with 3% OV225 on Gas Chrom Q (100–125 mesh), was used with a helium flow rate of 20 ml/min. Retention times (R_T) were determined relative to that of 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylmannitol. Ten ml of Bray's solution[16] and 5–10 μl samples were counted for radioactivity on a Nuclear Chicago scintillation counter.

Chromatography. — The following solvents were used (in volume ratios): (A)

butyl alcohol–pyridine–water (10:3:3); (B) butanone–acetic acid–water saturated boric acid (9:1:1)[17]; (C) propyl alcohol–acetic acid–water saturated with boric acid (5:2:1); (D) propyl alcohol–pyridine–ethanol–water (8:2:1:1) saturated with sodium arsenate crystals; and (E) benzene–ethanol (4:1). Butanone (Baker) was redistilled prior to its incorporation into Solvent B. Reducing sugars were located with the aniline hydrogen phthalate reagent[18], and alditols and sugars were detected with alkaline silver reagent[19]. Chromatograms of borate-complexed sugars were heated at 90° with three consecutive applications of anhydrous methanol prior to treatment with the alkaline silver reagent. Separation of mannitol and galactitol was accomplished on t.l.c. Sodium metaarsenate (10%) was incorporated into silica gel G and the chromatogram was developed with solvent D. The plates were visualized with alkaline permanganate[20]. The R_F values of arabinitol, mannitol, and galactitol were 0.37, 0.25 and 0.10, respectively. Solvent E was used on Brinkmann MN Sil N-HR (0.2 mm) precoated plates.

Hydrolyses. — Partial hydrolysis was carried out with 1–2 mg of oligosaccharide in 1 ml of 5mM HCl for 30 min at 70° in a sealed ampoule. Complete hydrolysis was effected in 0.1M HCl for 1 h at 100°. For preparative purposes, 100 mg of oligosaccharide was hydrolyzed in 5 ml of 0.5M HCl for 1 h at 100°. Methylated sugars were hydrolyzed on a steam bath for 3 h in 0.25M H_2SO_4. Enzymic hydrolyses were incubated for 1 h at 40° with 1 mg of oligosaccharide dissolved in 100 μl of invertase stock solution. Sigma invertase (sp. act. 0.116, grade VI) was dissolved in 0.05M acetate buffer pH 5.0 to yield a 1% stock solution.

Periodate oxidation. — Periodate oxidations of the oligosaccharides followed the conditions described for the Smith degradation by Gray and Ballou[21] and were complete within 24 h. The oxidations of tritium-labeled arabinitol and **4b** were stopped after 5 min by addition of ethylene glycol. Formyl-tetrahydrofolate synthetase was employed in determination of formic acid[22]. Although low formic acid values were obtained, the method was very sensitive and could be used on a small scale. In formaldehyde determinations, a modification of the 2,4-pentanedione–ammonia method[23] was used, with the sulfuric acid being omitted during reduction of periodate by arsenite.

Periodate oxidations of tritium-labeled sugars were treated in a similar manner. Both [1-^{3}H]-arabinitol and ^{3}H-**4b** were oxidized separately for 5 min. Each oxidation mixture was divided equally. One-half was reduced with sodium borohydride, the methanol thus produced was removed by evaporation under vacuum, and the residue was counted for radioactivity. The other half of the oxidation mixture was treated with the 2,4-pentanedione–ammonia reagent[23] to incorporate the [^{3}H]formaldehyde into a lutidine derivative. Instead of cooling the yellow incubation mixture, conc. aqueous ammonia was added and evaporated under vacuum. An acetone extract of the residue was chromatographed in solvent E. The plastic-backed t.l.c. plates were cut into 1-cm sections, and the radioactivity was counted. A yellow spot having R_F 0.5, representing 3,5-diacetyl-1,4-dihydro-2,6-lutidine, was strongly labeled from both the D-[1-^{3}H]-arabinitol and ^{3}H-**4b** preparations. The dye, which strongly quenched the

fluorescense in Bray's Solution, was decomposed by light, and the c.p.m. returned to the expected values after several days.

Methylated alditol acetates. — Lyophilized sugar (5 mg) was methylated in *N,N*-dimethylformamide with methyl iodide and silver oxide[24]. The silver oxide suspension was filtered through Celite on a Buchner funnel, which was then washed with benzene. The combined filtrate was washed first with 1% aqueous potassium cyanide and then with water. Following vacuum evaporation, the methylated oligosaccharide was hydrolyzed. The hydrolysate was neutralized with sodium hydrogen carbonate, evaporated to dryness under vacuum, and the partially methylated sugars were extracted from the residue with ether. After evaporation of the solvent, the sugars were reduced with sodium borohydride or borodeuteride at pH 7.5. Following decomposition of the excess borohydride ion, the partially methylated alditols were passed through a small column of AG 50-X8(H^+) resin. The effluent and washings from the ion-exchange resin were evaporated repeatedly, with addition of anhydrous methanol, and the residue was acetylated[25]. The partially methylated alditol acetate, derived from methyl β-D-fructofuranoside[26] by the foregoing procedure, gave a mixture of the mannitol and glucitol isomers, which appeared as a single peak on g.l.c. When this mixture was demethylated[27] with boron trifluoride and then fully acetylated, the glucitol and mannitol acetates were easily separated on g.l.c., and were detected in a 60:40 ratio. When such a mixture of isomers resulted from the methylation, hydrolysis and reduction of a substituted or unsubstituted fructose derivative, we have designated the product as a specifically methylated "hexitol".

Synthetic. — Preparation of 1-*O*-trityl-D-mannitol and 1,6-di-*O*-trityl-D-mannitol followed the procedure of Wolfrom *et al.*[28] but without the acetylation step. The monotrityl derivative was crystallized from petroleum ether (b.p. 60–90°) and ethanol, and recrystallized from toluene; m.p. 123.5–125.1°. The 1,6-di-*O*-trityl-D-mannitol was amorphous, m.p. 103°. When chromatographed in Solvent E and detected with 50% sulfuric acid in ethanol, one yellow spot was observed for the monotrityl derivative, whereas the ditrityl product had 25% of a monotrityl contaminant. Ten mg of each was methylated separately and then hydrolyzed in 2 ml of glacial acetic acid for 2 h at 100°. After the solution had been evaporated to 0.5 ml, water was added and the insoluble triphenylmethanol was removed by filtration. The aqueous solution was evaporated under vacuum and acetylated[25]. This procedure yielded 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylmannitol and a mixture of 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methyl and 1,6-di-*O*-acetyl-2,3,4,5-tetra-*O*-methylmannitol.

Standard 1,3,4-tri-*O*-methyl-D-fructose was prepared from levan[29], and 3,4,6-tri-*O*-methyl-D-fructose was prepared from inulin. The Hakomori method[30] was used to prepare tri-*O*-methylinulin, which was then permethylated with the Purdie reagent[24]. The tri-*O*-methylfructans were hydrolyzed by refluxing them with 0.05M sulfuric acid in 75% ethanol. The alcohol was gradually replaced with water by vacuum distillation at 7-h intervals, and the resulting solution was refluxed for 1 h longer. The solution was neutralized with sodium hydrogen carbonate and evaporated under vacuum. An ether extract of the residue was chromatographed on a cellulose

powder column by using butanone saturated with water, and the pure derivatives were isolated.

Honeydew. — A strain of *Sphacelia sorghi* McRae was isolated from ergot sclerotia grown on Combine Kafir 60-A Line *Sorghum vulgare* Pers. from Samaru-Zaria, Nigeria, during January 1969. A spore suspension of the parasite was inoculated on stigmas of sorghum in the greenhouse, and the plants were covered with plastic bags. The greenhouse was maintained at 20–30° and 40–80% humidity. After 14 h, the bags were removed. Honeydew began to appear on the florets 6 days after inoculation, and was collected in disposable syringes up to 10 days later. Spores and pollen were removed from the honeydew by diluting it with distilled water and centrifuging the solution at 15,000 × g for 10 min.

Media. — Both stationary and shake cultures were grown on the TS5 sucrose medium[31], with or without 1% of alditol. Fifty ml of broth was used in 250-ml Erlenmeyer flasks for shake cultures, and 25 ml of broth was used for stationary culture.

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