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STUDIES OF OLIGOSACCHARIDE LINKAGES BY SIMULTANEOUS DETERMINATION OF COMPONENT ALDEHYDES IN DIALDEHYDE FRAGMENTS AS (2,4-DINITROPHENYL)HYDRAZONES*

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ABSTRACT

The component aldehydes in dialdehyde fragments formed by periodate oxidation of oligosaccharides were converted quantitatively into the corresponding (2,4-dinitrophenyl)hydrazones by the simple procedure of treatment with excess (2,4-dinitrophenyl)hydrazine hydrochloride in 1,2-dimethoxyethane. Then, by chromatographic separation of the hydrazones on a small column of silica gel and subsequent spectrophotometric analysis, it was possible to determine the position of glycosidic substitution in μmolar amounts of various types of glucobioses, oligosaccharides of senega, and some synthetic (1→6)-β-D-gluco-oligosaccharides.

INTRODUCTION

Periodate oxidation of oligo- and poly-saccharides produces dialdehyde fragments that are composed of various aldehydes linked through hemiacetal bonds. Determination of the aldehydic compositions of such dialdehydes provides information useful for linkage analysis that cannot be obtained from studies on periodate consumption alone. Therefore, studies have been made by gas-chromatographic analysis of the products of Smith degradation[1–3]. However, the methods used have limitations, because the oxidation products must be subjected to a number of chemical treatments (borohydride reduction, hydrolysis, oximation, and trimethylsilylation) before gas-chromatographic determination. Some workers[4–8] have converted the component aldehydes into the corresponding phenylosazones for the elucidation of polysaccharide structures. These studies were made on a macro scale and were not quantitative, but they had the merit that the number of chemical

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processes involved was decreased to two (hydrolysis and formation of the phenylosazones). We have reinvestigated the reactions of dialdehydes with various phenylhydrazine derivatives and have found that (2,4-dinitrophenyl)hydrazine is the most suitable reagent for linkage analysis. With this reagent, formation of hydrazones proceeds readily at room temperature with quantitative yields, and osazones are formed only under drastic conditions[9]. Hydrolysis of acetal and hemiacetal bonds occurred concurrently with hydrazone formation, and the hydrazones of component aldehydes were obtained quantitatively. The use of a spectrophotometric technique, coupled with chromatographic separation on a column of silica gel, made it possible to perform the analysis on a μmolar scale. Fundamental analytical data obtained in this way with glycoside and dialdehyde models have been published[10]. This article demonstrates the applicability of the method for determination of the position of glycosidic substitution in some oligosaccharides.

RESULTS AND DISCUSSION

Dialdehydes obtained from oligosaccharides by periodate oxidation are considered to be equilibrium mixtures of hemialdals and free aldehydes. When these fragments are allowed to react with an excess of phenylhydrazine in dilute acetic acid at room temperature, the free carbonyl groups condense with this reagent to yield phenylhydrazones. The structures of these hydrazones are not simple, but at least their interglycosidic linkages (derived from the precursor oligosaccharides) are considered to remain intact. In the Barry degradation[4–8], these hydrazones are treated further (under reflux) with excess phenylhydrazine in dilute acetic acid to obtain the phenylosazones of the component aldehydes. The conditions required for osazone formation are so drastic that considerable proportions of the hydroxyaldehydes, especially of those having several carbon atoms, are decomposed during the reaction.

(2,4-Dinitrophenyl)hydrazine offers advantages over phenylhydrazine, because the hydrochloride of the former hydrazine cleaves interglycosidic linkages and facilitates hydrazone formation, not only from carbonyl groups formed by periodate oxidation, but also from those liberated by hydrolysis of interglycosidic linkages. Thus, the component aldehydes in dialdehyde fragments may be converted quantitatively into the corresponding (2,4-dinitrophenyl)hydrazones, simply by mixing the fragments with excess (2,4-dinitrophenyl)hydrazine hydrochloride in 1,2-dimethoxyethane. No intermediate isolation procedure, such that needed in the Barry degradation, is required. Under similar conditions, phenylhydrazine hydrochloride also gives the hydrazones of the component aldehydes. However, the reaction is slow and incomplete, as shown by thin-layer chromatographic examination using a model compound, 7,9-dihydroxy-6α-methoxy-2-phenyl-*trans*-*m*-dioxano-[5,4-e]-(1,4)-dioxepane hydrate, a crystalline oxidation product obtained from methyl 4,6-*O*-benzylidene-α-D-glucopyranoside. The superiority of (2,4-dinitrophenyl)hydrazine for hydrazone formation seems to be due to its lower basicity, resulting from the electron-attracting activity of the nitro groups. Introduction of nitro groups may also favor

osazone formation in strongly acidic media at elevated temperature[9], but little, if any, osazone was formed under the present conditions.

The analytical conditions used are based on results given in a previous report[10]. Although the configurational effect on the rate of oxidation should not be ignored, our data indicate that the amounts of periodate consumed by common glycosides of natural origin reach theoretical values (within the limits of experimental error) on oxidation for 3 h at 25°.

Linkage analysis of glucobioses. — Five types of interglycosidic linkages are possible for glucobioses having pyranose rings, namely 1→1, 1→2, 1→3, 1→4, and 1→6. Under conditions wherein normal oxidation takes place quantitatively, the (1→2)- and (1→3)-linked bioses should both consume 3 moles of periodate per mole. Similarly, the (1→1)- and (1→4)-linked bioses should both consume 4 moles of periodate per mole. Hence, determination of the positions of glycosidic substitution is impossible from results on periodate consumption alone.

On the other hand, the kind and number of molecules of component aldehydes present in dialdehyde fragments vary with the type of linkage. One molecule of glyoxal is formed from the C-1–C-2 part of every non-reducing D-glucose residue, and so this particular aldehyde may be adopted as an internal standard. The upper columns of Table I give the molar proportions of the hydroxyaldehydes, relative to glyoxal, expected for each type of biose. Each type gives one molecule of D-glyceraldehyde from the C-4–C-5–C-6 part of the non-reducing D-glucose residue. Each (1→1)-, (1→2)-, and (1→6)-linked biose produces an additional molecule of D-glyceraldehyde from the reducing D-glucose residue. Of these three types of biose, the (1→1)-linked bioses give two molecules of glyoxal, but the (1→2)- and (1→6)-linked bioses produce only one, so that the D-glyceraldehyde:glyoxal molar ratios for the (1→1)-, (1→2)-, and (1→6)-linked bioses are calculated as 1, 2, and 2, respectively. The (1→2) and (1→6) linkages can be differentiated, as the (1→2)-linked bioses yield a unique aldehyde component, namely 2-hydroxymalonaldehyde, from the C-1–C-2–C-3 part of the reducing D-glucose residue. From the (1→3)- and (1→4)-linked bioses, D-arabinose (from the C-2–C-3–C-4–C-5–C-6 part) and D-erythrose (from the C-3–C-4–C-5–C-6 part) are formed at the reducing D-glucose residue, respectively, each in an equimolar ratio to glyoxal. Thus, each type of biose generates a distinct pattern of aldehyde distribution, and consequently only one linkage-type is possible for a given pattern.

Eight glucobioses having various types of interglycosidic linkages were oxidized with sodium metaperiodate at 25°. After 3 h, the component aldehydes in the dialdehyde fragments were determined by the (2,4-dinitrophenyl)hydrazine method. The results are summarized in the lower columns of Table I. The molar ratio of D-glyceraldehyde (the most common hydroxyaldehyde) relative to glyoxal was determined accurately, except for the pair of (1→2)-linked bioses (kojibiose and sophorose). For maltose and cellobiose, the component D-erythrose was found in equimolar ratio to glyoxal. The experimental errors were within one per cent of the theoretical values for each biose. The amount of D-arabinose found for laminarabiose, a (1→3)-linked biose,

TABLE I

MOLAR RATIOS OF ALDEHYDES IN THE PRODUCTS OF PERIODATE OXIDATION OF GLUCOBIOSES

Linkage	*Name of glucobiose*	*Molar ratio of aldehyde*			
		D-*Glyceraldehyde: glyoxal*	D-*Erythrose: glyoxal*	D-*Arabinose: glyoxal*	*2-Hydroxymalonaldehyde: glyoxal*
Theoretical					
1→1		1	0	0	0
1→2		2	0	0	1
1→3		1	0	1	0
1→4		1	1	0	0
1→6		2	0	0	0
Found					
α,α-(1→1)	Trehalose	0.99	0.00	0.00	0.00
α-(1→2)	Kojibiose	1.68	0.01	0.00	detected
β-(1→2)	Sophorose	1.85	0.00	0.00	detected
β-(1→3)	Laminarabiose	1.04	0.00	0.91	0.00
α-(1→4)	Maltose	1.01	0.99	0.00	0.00
β-(1→4)	Cellobiose	1.01	1.00	0.00	0.00
α-(1→6)	Isomaltose	2.01	0.01	0.01	0.00
β-(1→6)	Gentiobiose	2.02	0.00	0.00	0.00

was slightly smaller than the expected value, probably because of contamination of the sample with impurities. Kojibiose and sophorose, especially the former, gave considerably lower D-glyceraldehyde:glyoxal molar ratios than expected. It has been suggested that, in the pyranose–aldehydo sugar equilibria of (1→2)-linked glucobioses, the proportion of the latter forms is relatively high[11]. The participation of the aldehydo forms may cause partial cleavage of C-3–C-4, C-4–C-5, and C-5–C-6 bonds at reducing D-glucose residues, decreasing the amounts of D-glyceraldehyde formed. However, it should be noted that the bis(hydrazone) of 2-hydroxymalonaldehyde, which is characteristic of 1→2 linkages, gave a red color having an absorption maximum at 485 nm on addition of alkali. Determination of this component was not possible because an authentic specimen was not available.

Confirmation of the structures of the oligosaccharides found in senega. — During a survey of the distribution of oligosaccharides in plants, we found melibiose, sucrose, raffinose, and stachyose in the roots of *Polygala senega* L. *var. latifolia*[12]. In addition to these common oligosaccharides, we also isolated two isomeric disaccharides (**1** and **2**) containing 1,5-anhydro-D-glucitol (polygalitol), to which the structures 1,5-anhydro-2-*O*-β-D-galactopyranosyl-D-glucitol and 1,5-anhydro-6-*O*-α-D-glucopyranosyl-D-glucitol, respectively, were assigned on the basis of results of methylation analysis and enzymic hydrolysis, as well as p.m.r. and mass spectroscopy.

The structures of these oligosaccharides were confirmed by the (2,4-dinitrophenyl)hydrazine method. As summarized in Table II, melibiose, a (1→6)-linked biose, gave amounts of glyoxal and D-glyceraldehyde approximately equal to those

TABLE II

DETERMINATION OF ALDEHYDES IN THE PRODUCTS OF PERIODATE OXIDATION OF OLIGOSACCHARIDES FROM *Polygala senega* L. *var. latifolia*

Oligosaccharide	*Aldehyde (mole/mole of oligosaccharide)*					
	Glyoxal		D-*Glyceraldehyde*		*Hydroxypyruvaldehyde*	
	Theoretical	*Found*	*Theoretical*	*Found*	*Theoretical*	*Found*
Melibiose	1	0.98	2	1.96	0	0.00
Sucrose	1	0.99	2	1.89	1	0.93
Raffinose	2	1.95	3	2.89	1	0.96
Stachyose	3	2.93	4	3.89	1	0.95

expected. The amounts of glyoxal and D-glyceraldehyde found for sucrose, raffinose, and stachyose also agreed with the theoretical values, within the limits of experimental error. Hydroxypyruvaldehyde is a characteristic dicarbonyl component formed from the C-1–C-2–C-3 part of the D-fructose residue. Its bis(hydrazone), identical with the osazone of glyceraldehyde, has an intense color and an absorption maximum at 435 nm, bathochromically shifted from those of the hydrazones of other hydroxyaldehydes.

The disaccharides **1** and **2** were found by chromatographic analysis to be D-galactopyranosyl and D-glucopyranosyl derivatives of 1,5-anhydro-D-glucitol, respectively. As shown in Table III, linkage analysis by the (2,4-dinitrophenyl)-hydrazine method indicated that disaccharide **1** gave a D-glyceraldehyde:glyoxal molar ratio of approximately 3:1. Only a 1→2 linkage is consistent with these data. Similarly, the D-glyceraldehyde:glyoxal molar ratio for disaccharide **2** was approximately 2:1. In this instance, 1 mole of glycolaldehyde per mole of glyoxal was also found. These findings confirmed that disaccharide **2** has a 1→6 linkage.

TABLE III

MOLAR RATIOS OF ALDEHYDES IN THE PRODUCTS OF PERIODATE OXIDATION OF DISACCHARIDES, **1** AND **2**

Linkage	*Molar ratio of aldehyde*			
	Glycolaldehyde: glyoxal	D-*Glyceraldehyde: glyoxal*	D-*Erythrose or* D-*threose: glyoxal*	*1,5-Anhydro-*D-*glucitol:glyoxal*
Theoretical				
1→2	0	3	0	0
1→3	0	1	0	1
1→4	1	1	1	0
1→6	1	2	0	0
Found				
1	0	2.95	0.00	0.00
2	0.94	1.92	0.00	0.00

Confirmation of the absence of the 1→4 linkage in synthetic (1→6)-β-D-gluco-oligosaccharides. — Recently we synthesized (1→6)-β-D-gluco-oligosaccharides having a chain length of up to hexaose by block condensation[13]. In these syntheses, 1,2,3,4,2′,3′,4′-hepta-*O*-acetylgentiobiose was used as an intermediate oligosaccharide block. A portion of this compound was found to undergo 4→6 acetyl migration during Koenigs–Knorr condensation of it with acetylated (1→6)-β-D-gluco-oligosaccharide glycosyl bromides, and so the products might contain a 1→4 linkage. However, on linkage analysis of the products, no D-erythrose was detected in the oxidation products from the biose, triose, and tetraose, and the yields of glyoxal and D-glyceraldehyde were quantitative (Table IV). These results exclude the presence of the 1→4 linkage as a possibility. This may be attributed to the greater reactivity of the primary hydroxyl group in the Koenigs–Knorr condensation.

TABLE IV

DETERMINATION OF ALDEHYDES IN THE PRODUCTS OF PERIODATE OXIDATION OF SYNTHETIC (1→6)-β-D-GLUCO-OLIGOSACCHARIDES

(1→6)-β-D-*Gluco-oligosaccharide*	*Aldehyde (mole/mole of oligosaccharide)*					
	Glyoxal		D-*Glyceraldehyde*		D-*Erythrose*	
	Theoretical	*Found*	*Theoretical*	*Found*	*Theoretical*	*Found*
Biose	1	0.97	2	1.96	0	0.00
Triose	2	1.96	3	2.94	0	0.00
Tetraose	3	2.98	4	3.89	0	0.00

EXPERIMENTAL

Materials. — Crystalline samples of trehalose dihydrate, maltose monohydrate, and cellobiose were obtained commercially. Samples of kojibiose[11], sophorose[11], isomaltose[14], and (1→6)-β-D-gluco-oligosaccharides[13] were synthesized in our laboratory. Laminarabiose was obtained as a syrup by a reported method[15], and purified by paper chromatography. Melibiose, sucrose, raffinose pentahydrate, stachyose tetrahydrate, and the disaccharides **1** and **2** were isolated from roots of *Polygala senega* L. *var. latifolia*, and purified by charcoal-column chromatography[12]. The preparation and physical data for authentic (2,4-dinitrophenyl)hydrazones of various aldehydes were reported previously[10,16]. Data on the authentic bis(hydrazone) of hydroxypyruvaldehyde were not reported previously. This material was prepared by osazonization of DL-glyceraldehyde as described in the literature[9]. It decomposed gradually at above ~280° without melting.

Anal. Calc. for $C_{15}H_{12}N_8O_9$: C, 40.18; H, 2.70; N, 25.00. Found: C, 40.12; H, 2.87; N, 25.32.

Periodate oxidation of oligosaccharides. — Each oligosaccharide sample, containing ~200 μg of total monosaccharide(s), was dissolved in 1% sodium meta-

periodate (0.2 ml), and the solution was kept in the dark for 3 h at 25°. Under these conditions, the amounts of periodate consumed by methyl α-D-galactopyranoside, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, and methyl α-D-mannopyranoside, as measured titrimetrically[17], were 1.92, 2.03, 1.95, and 1.91 moles per mole of glycoside, respectively.

Determination of the component aldehydes in dialdehyde fragments. — The procedure described in the previous paper[10] was slightly modified. The oxidation mixtures obtained were each deionized by passage through a small column containing Amberlite IR-120 (H^+ form) and Amberlite IRA-410 (HCO_3^- form). The eluate and washing fluids (~1 ml) were combined and evaporated to dryness, and then a saturated solution (0.2 ml) of (2,4-dinitrophenyl)hydrazine hydrochloride in 1,2-dimethoxyethane was added. After 3 h, the mixture was applied to a small column (1.0 cm i.d., 10 cm long) of silica gel (Mallinckrodt Chemical Works, 5.0 g) impregnated with chloroform. The column was eluted with chloroform (60 ml), chloroform–methanol (20:1, v/v; 40 ml), and finally with chloroform–methanol (10:1, v/v; 80 ml). The colored bands separated were fractionated, and each fraction was identified by comparison with an authentic specimen of the hydrazone on a t.l.c. plate (Merck Silica Gel Plate No. 5721/0025), developed with chloroform–methanol systems. The mobilities of the hydrazones were all given in the previous paper[10], except for those of two dicarbonyl components, 2-hydroxymalonaldehyde [0.48 in 10:1 (v/v) chloroform–methanol; 0.66 in 5:1 (v/v) chloroform–methanol] and hydroxypyruvaldehyde [0.59 in 10:1 (v/v) chloroform–methanol; 0.77 in 5:1 (v/v) chloroform–methanol]. All fractions, except those of dicarbonyl components, were diluted to 25.0 ml with methanol, and their absorbances were read at 350 nm. Each of the glyoxal and hydroxypyruvaldehyde fractions was evaporated to dryness. The residue was dissolved in aqueous 20% dimethyl sulfoxide (6.00 ml), and a 12% aqueous ethanolic (1:4, v/v) solution of potassium hydroxide (1.00 ml) was added. The absorbances were read at 576 and 545 nm, respectively, and the amount of each fraction was calculated from its molar absorptivity.

Reaction of 7,9-dihydroxy-6α-methoxy-2-phenyl-trans-m-*dioxano*-[*5,4e*]-(*1,4*)-*dioxepane hydrate with phenylhydrazine hydrochloride.* — This model dialdehyde (3.2 mg, 10 μmoles) was dissolved in methanol (1 ml), and phenylhydrazine hydrochloride (14 mg, 100 μmoles) was added. After 3 h, the mixture was examined by t.l.c. on an alumina plate (Wako Alumina B-5), developed with ether–hexane (1:1, v/v). Three spots having R_F 0.77, 0.21, and 0.88, were detected, together with the spot of unreacted dialdehyde (R_F 0.00). The R_F values of the authentic phenylhydrazones of glyoxal, D-erythrose, and benzaldehyde were 0.76, 0.21, and 0.88, respectively.

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