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INTERACTION OF (1→4)- AND (1→6)-LINKED DISACCHARIDES WITH THE FENTON REAGENT UNDER PHYSIOLOGICAL CONDITIONS

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ABSTRACT

Reactions of (1→4)- and (1→6)-linked disaccharides, mainly of maltose and isomaltose, with the Fenton reagent under physiological conditions were studied. Chemical characterization of oxidation products was conducted by g.l.c. and g.l.c.–m.s. of their trimethylsilyl derivatives, and the results demonstrated that (1→6)-linked disaccharides are more reactive with the hydroxyl radical (·OH) generated by the Fenton reagent than (1→4)-linked disaccharides. About 35–40% of (1→6)- and 15–20% of (1→4)-linked disaccharides were oxidatively degraded to smaller molecules after incubation for 24 h. Of the four disaccharides examined, namely, maltose, isomaltose, cellobiose, and gentiobiose, the α-(1→6)-linked disaccharide isomaltose exhibited the highest reactivity, whereas the β-(1→4)-linked disaccharide cellobiose showed the lowest. These results suggest the existence of a relationship between the configuration of the glycosidic linkage and the reactivity with ·OH in aqueous solution.

INTRODUCTION

The Fenton system [H_2O_2–Fe(II)] has been studied with increasing vigor and frequently applied to oxidation reactions, because of its intensive reactivity with most organic compounds and its intimate relationship to the oxidative reactions occurring in biological systems[1,2]. It has been considered that the Fenton system involves cleavage of the HO–OH bond, catalyzed by a metal ion (M). It generates the potent oxidizing species, the hydroxyl radical (·OH), and its chemical equation may be represented as follows[3–5].

$$M_n + H_2O_2 \rightarrow M_{n+1} + \cdot OH + OH^- \qquad (1)$$

In general, iron [Fe(II)] has been used in the Fenton reagent, but preliminary experiments suggested that copper ion [Cu(II)] is a more effective catalyst than Fe(II) under physiological conditions (unpublished data), and we have therefore applied the H_2O_2–Cu(II) system as a modified Fenton reagent in this investigation.

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The reaction mechanism of the system may be represented by the following equations[6].

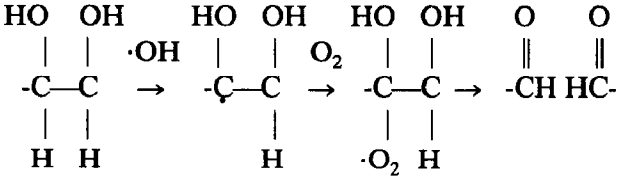
$$Cu(II) + H_2O_2 \rightarrow Cu(I) + HO_2^{\cdot} + H^+ \quad (2)$$

$$HO_2^{\cdot} \rightarrow H^+ + O_2^{\cdot-} \quad (3)$$

$$Cu(II) + O_2^{\cdot-} \rightarrow Cu(I) + O_2 \quad (4)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {\cdot}OH + OH^- \quad (5)$$

Hydroxyl radical has been regarded as a potent oxidant, and it acts on several biological components by hydroxylation and hydrogen abstraction. Especially, the reaction of ·OH with sugars is initiated by hydrogen abstraction, and this is followed by oxidative C–C cleavage of sugar molecules under aerated conditions which can cause depolymerization of sugars and an increase of new carbonyl compounds (see reaction *6*)[7,8].



(6)

In fact, a considerable decrease in viscosity and generation of reducing groups were observed during the reaction of dextran with the H_2O_2–Fe(II) system[9]. Moreover, carbon-centered radicals induced by hydrogen abstraction from neutral and acidic polysaccharides were characterized by e.s.r. experiments employing a flow system in H_2O_2 and Ti(III) solutions[10].

On the basis of these experiments, we have studied the reactions of oligosaccharides and polysaccharides with ·OH under physiological conditions. In the course of this investigation, we observed that the degree of polymerization and the kind of glycosidic linkages of the sugar units affected the reactivities with ·OH generated *via* the modified Fenton system [H_2O_2–Cu(II)][11,12]. In this report, we demonstrate that, for these sugars, the (1→6)-linked disaccharides are more reactive than the (1→4)-linked, and that the α-linked are slightly more reactive than the β-linked.

RESULTS AND DISCUSSION

As shown in Table I, remarkable differences in the reactivity of amylose (mol. wt. 3×10^3) and dextran (mol. wt. 5.8×10^3) in the H_2O_2–Cu(II) system were observed. The increase in reducing sugars corresponded to the oxidation of

TABLE I

OXIDATION OF AMYLOSE AND DEXTRAN WITH THE FENTON REAGENT[a]

Glycan	*Reducing sugars (μg/mL)*[b]		
	Control	*After treatment with Fenton reagent*	*Increment*
Amylose	49.9	88.8	+38.9
Dextran	31.0	82.1	+51.1

[a]Reactions were carried out in 25 mL of phosphate buffer containing 10 mg of the polysaccharide (0.04%, w/v), mM hydrogen peroxide, and 10μM copper ion for 6 h at room temperature. [b]Reducing sugars were measured colorimetrically by the Park–Johnson method[17], and the values were calculated as the amount of D-glucose.

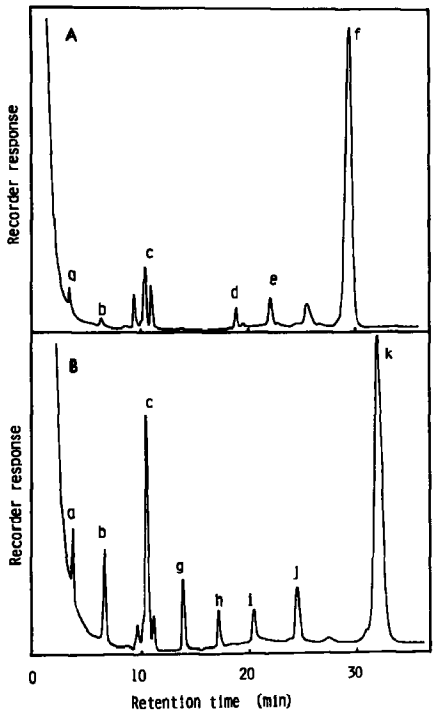
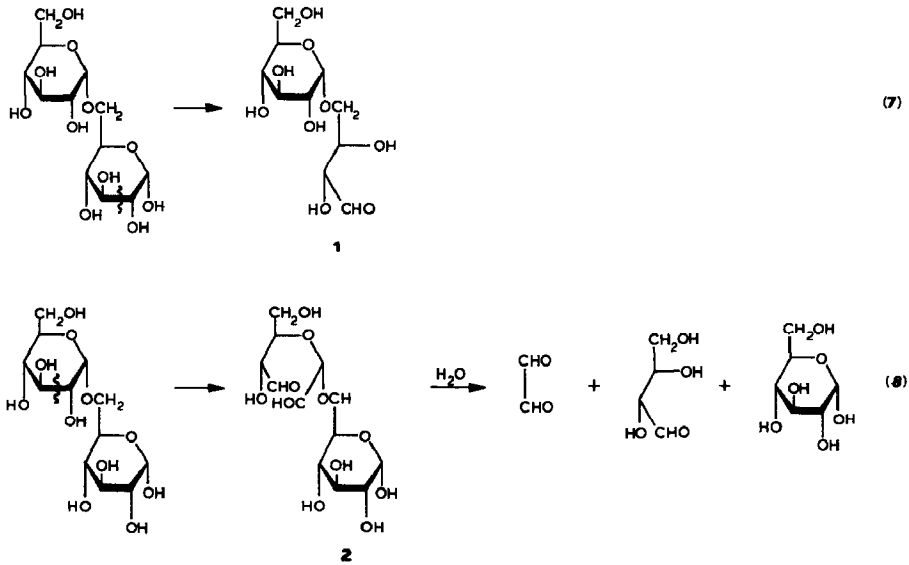


Fig. 1. Trimethylsilyl derivatives of compounds from the oxidation of maltose (A) and isomaltose (B). [Peaks: trimethylsilyl derivatives of D-erythritol (peak a), D-arabinitol (peak b), D-glucitol (peak c), α-D-Glc*p*-(1→2)-D-erythritol or α-D-Glc*p*-(1→3)-D-threitol (peak d), α-D-Glc*p*-(1→3)-D-arabinitol or α-D-Glc*p*-(1→4)-D-xylitol (peak e), α-D-Glc*p*-(1→4)-D-glucitol (peak f), α-D-Glc*p*-(1→2)-D-glycol (peak g), α-D-Glc*p*-(1→3)-D-glycerol (peak h), α-D-Glc*p*-(1→4)-D-erythritol (peak i), α-D-Glc*p*-(1→5)-D-arabinitol (peak j), and α-D-Glc*p*-(1→6)-D-glucitol (peak k). Peaks f and k correspond to maltose and isomaltose, respectively].

the oligosaccharides with ·OH, followed by the formation of reducing terminal groups (reaction *6*). We speculated from this result that the differences in the reactivity of amylose and dextran may be attributed to the effect of the (1→4) and (1→6) bonds. Therefore, in order to explain their reactivities by means of chemical evidence, maltose and isomaltose were used as model examples of α-(1→4)- and α-(1→6)-linked disaccharides, respectively, in the following experiments.

Fig. 1 depicts the gas–liquid chromatogram of trimethylsilyl derivatives of the compounds formed in the oxidation of maltose and isomaltose. It was ascertained that isomaltose is more reactive than maltose with ·OH. Chemical characterization of the derivatives was carried out by g.l.c. and g.l.c.–m.s. It was estimated from the following that D-glucitol (peak c) was mainly derived from the reducing terminals of the D-glucose residues. As shown in reactions *7* and *8*, if the reaction was initiated by abstraction of hydrogen from C-2 or C-3 in the reducing moiety of the disaccharide, oxidative C–C bond cleavages could afford the stable, oxidized disaccharide (**1**) (see reaction *7*). However, oxidation of the nonreducing moiety could produce the oxidized disaccharide (**2**), which would undergo rapid hydrolysis to D-glucose, glyoxal, and D-erythrose (reaction *8*).



A similar tendency was observed in the reaction of the β-linked disaccharides, cellobiose [β-D-Glc*p*-(1→4)-D-Glc*p*] and gentiobiose [β-D-Glc*p*-(1→6)-D-Glc*p*], with the Fenton reagent (see Fig. 2).

Identification of the oxidized disaccharides (peaks d and e derived from maltose, and peaks g, h, i, and j derived from isomaltose in Fig. 1) was accomplished from their mass-fragmentation (see Figs. 3–5). As suggested in reaction *7*, the nonreducing moieties of the peaks (d, e, g, h, i, and j) are presumed to be α-D-glucosyl groups and the reducing moieties to be derived by degradation of the rest of the

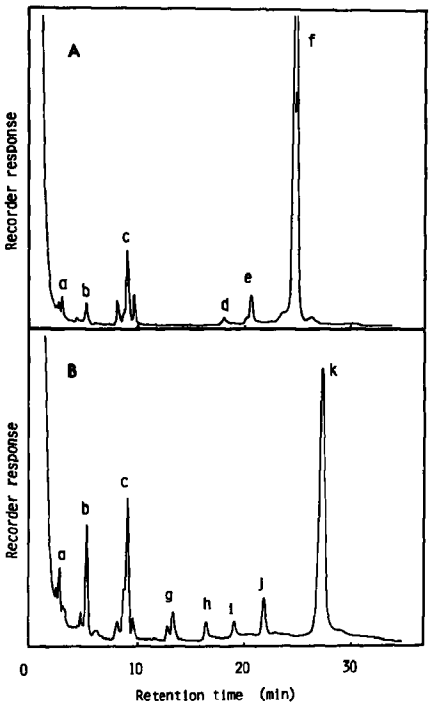


Fig. 2. Trimethylsilyl derivatives of compounds from the oxidation of cellobiose (A) and gentiobiose (B). [Peaks: trimethylsilyl derivatives of D-erythritol (peak a), D-arabinitol (peak b), D-glucitol (peak c), β-D-Glc*p*-(1→2)-D-erythritol or β-D-Glc*p*-(1→3)-D-threitol (peak d), β-D-Glc*p*-(1→3)-D-arabinitol or β-D-Glc*p*-(1→4)-D-xylitol (peak e), β-D-Glc*p*-(1→4)-D-glucitol (peak f), β-D-Glc*p*-(1→2)-D-glycol (peak g), β-D-Glc*p*-(1→3)-D-glycerol (peak h), β-D-Glc*p*-(1→4)-D-erythritol (peak i), β-D-Glc*p*-(1→5)-D-arabinitol (peak j), and β-D-Glc*p*-(1→6)-D-glucitol (peak k). Peaks f and k correspond to cellobiose and gentiobiose, respectively].

disaccharide. Although the trimethylsilyl derivatives of their alditols did not give a molecular ion and their mass-fragmentation patterns were extremely similar, a specific fragmentation as shown in Scheme 1 suffices to characterize their structures[13]. In the series of fragments, the fragment ion 3 ($Me_3SiO–CH=O^+–R$) was the most characteristic one in each spectrum. The fragment ions corresponding to $Me_3SiO–CH=O^+–R$ were *m/z* 235 (peak g), 337 (peak h), 439 (peaks d and i), and 541 (peaks e and j). Consequently, the structures of alditols derived from oxidized disaccharides in Fig. 1 were characterized as the trimethylsilyl derivatives of α-D-Glc*p*-(1→2)-D-erythritol or α-D-Glc*p*-(1→3)-D-threitol (peak d), α-D-Glc*p*-(1→3)-D-arabinitol or α-D-Glc*p*-(1→4)-D-xylitol (peak e), α-D-Glc*p*-(1→2)-glycol (peak g), α-D-Glc*p*-(1→3)-D-glycerol (peak h), α-D-Glc*p*-(1→4)-D-erythritol (peak i), and α-D-Glc*p*-(1→5)-D-arabinitol (peak j). Among the series of oxidized products, peaks g and h, derived from isomaltose and gentiobiose, appear to be characteristic of (1→6)-linked disaccharides. The compounds corresponding to peaks g and h are formed from (1→6)-linked disaccharides by way of the methylene group containing

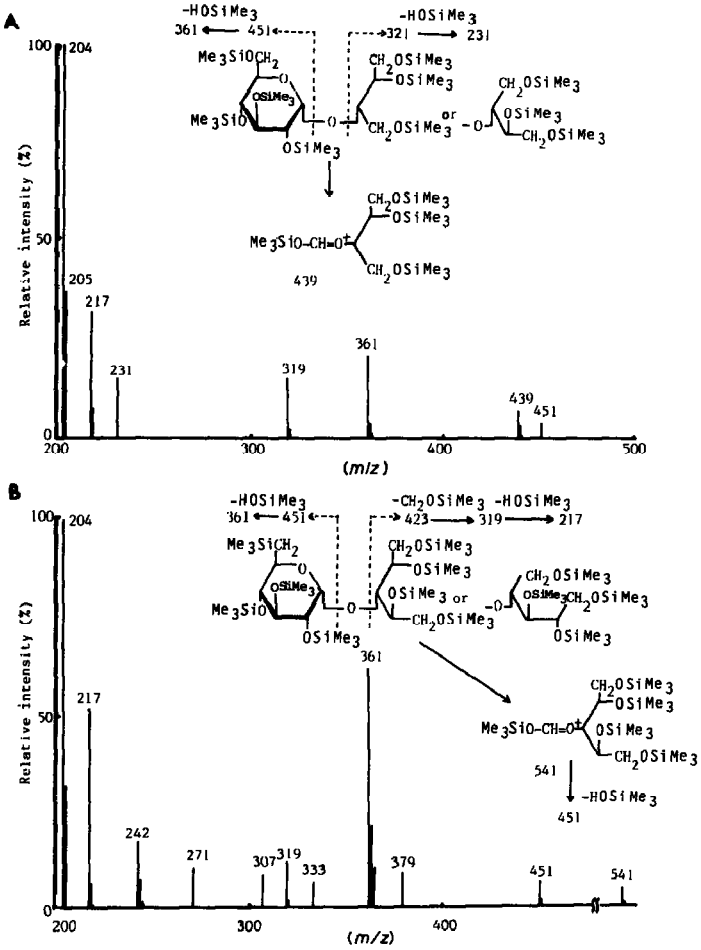


Fig. 3. Mass spectra of peaks d (A) and e (B) in Fig. 1.

C-6. With the (1→4)-linked disaccharides, compounds corresponding to peaks g and h are not found because, if produced, they would be hydrolyzed to D-glucose and a fragment of the oxidized monosaccharides.

The yields of oxidized products are summarized in Table II, and it was ascertained that α- and β-(1→6)-linked disaccharides are more reactive than (1→4)-linked ones. Moreover, cellobiose exhibited the lowest reactivity with ·OH, and the result enabled us to speculate on the relationship to the most stable and rigid polysaccharide, namely, cellulose. The cellulose chains form strong intramolecular hydrogen-bonds and have a stable conformation, and such a characteristic property may affect the protective ability against oxidation.

A somewhat higher reactivity of (1→6)-linked disaccharides might be attributable to their conformations in aqueous solution. As shown in Fig. 6, in general, the linkage orientation of (1→4)-linked D-glucosides is defined by two

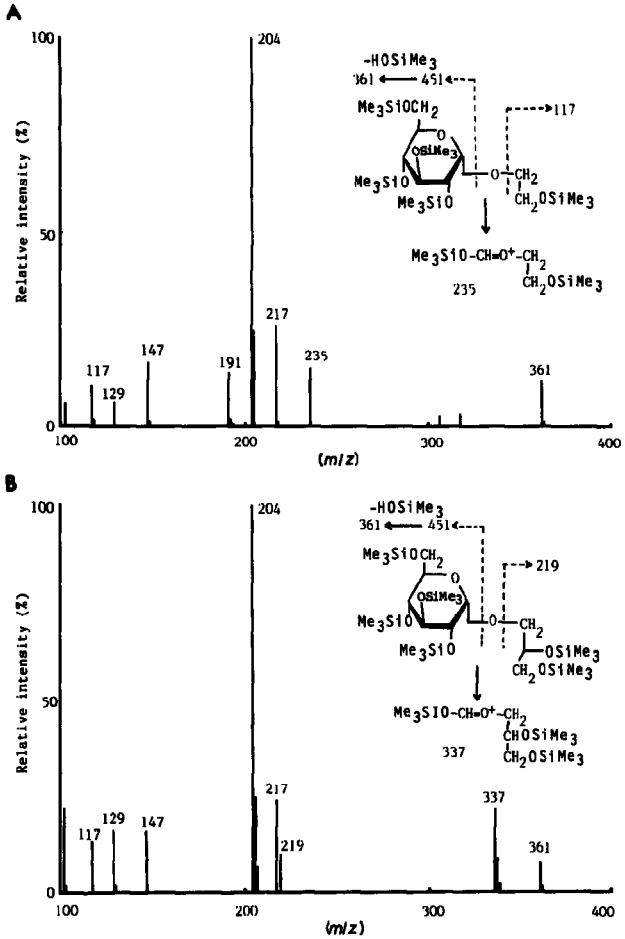


Fig. 4. Mass spectra of peaks g (A) and h (B) in Fig. 1.

dihedral angles (ϕ and ψ) as in the structure **4**, and conformational mobility is considerably restricted by the hydrogen bond. On the other hand, the linkage orientation of (1→6)-linked D-glucosides is defined by three dihedral angles (ϕ, ψ, and ω), as in structure **5**, permitting increased freedom to adopt a wide variety of orientations[14]. Consequently, it seems probable that the Fenton reagent (presumably the copper ion) may be more effectively accessible to (1→6)-linked than to (1→4)-linked D-glucosides, and the former will be susceptible to oxidative reaction with ·OH generated *in situ*. It is well known that Cu(II) shows a higher ability to form complexes with various sugars than do other metal cations[15,16]. Therefore, the differing reactivities of (1→4)- and (1→6)-linked disaccharides with the Fenton reagent may arise from their ability to form complexes with Cu(II).

In this report, we have undertaken the chemical characterization of oxidized products derived from disaccharides, and we conclude that the results are in accord

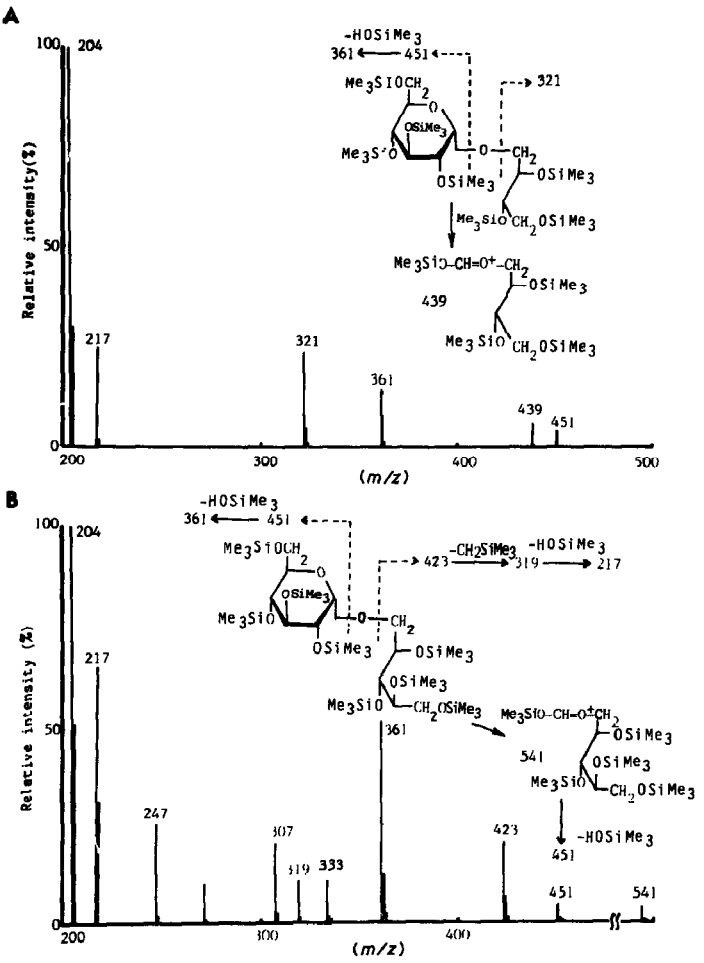
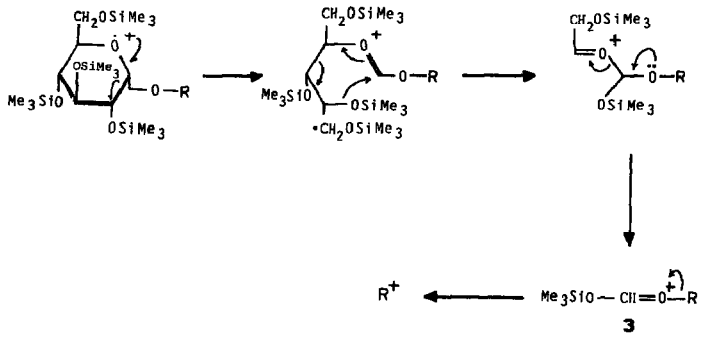


Fig. 5. Mass spectra of peaks i (A) and j (B) in Fig. 1.



Scheme 1. Typical route of fragmentation of oxidized disaccharides.

TABLE II

OXIDATION OF DISACCHARIDES WITH THE FENTON REAGENT[a]

Disaccharide	*Link*	*Yield of oxidized products (%)*
Maltose	α-(1→4)	19.7
Cellobiose	β-(1→4)	13.4
Isomaltose	α-(1→6)	39.4
Gentiobiose	β-(1→6)	34.8

[a]The reactions were carried out in 25 mL of phosphate buffer containing mM sugar, 10mM hydrogen peroxide, and 50μM copper for 24 h at room temperature.

with the analytical data obtained from the reactions of amylose and dextran with the Fenton reagent.

EXPERIMENTAL

Materials. — Maltose and isomaltose were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama), and cellobiose and gentiobiose from Nakarai Chemicals Ltd. (Osaka). Hydrogen peroxide (31% w/v) was obtained from Mitsubishi Gas Co. (Tokyo), and was stored at 4°. Other reagents were of the highest grade commercially available.

Reaction of amylose and dextran with the Fenton reagent. — The reactions were carried out in 25 mL of 67mM phosphate buffer (pH 7.2) containing mM hydrogen peroxide and 10μM $CuSO_4$ as the Fenton reagent, with 10 mg of the polysaccharide (0.04%). The reaction mixtures were kept for 24 h at room temper-

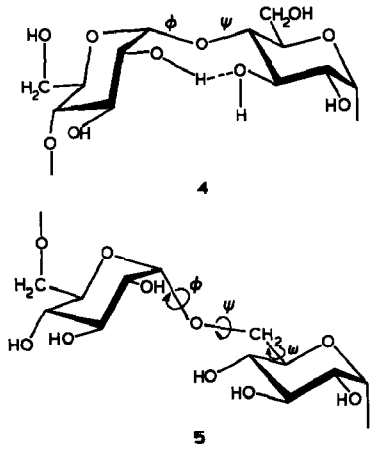


Fig. 6. Conformations of (1→4)- and (1→6)-linked disaccharides (**4** and **5**, respectively) in aqueous solution.

ature, and the reactions were terminated by addition of 10^4 units of catalase to decompose the remaining hydrogen peroxide. Oxidation of sugars was measured, as the increase in reducing power, by the Park–Johnson method[17].

Reaction of disaccharides with the Fenton reagent. — The reactions were conducted in 25 mL of 67mM phosphate buffer (pH 7.2) containing 5mM hydrogen peroxide and 50μM $CuSO_4$ as the Fenton reagent, and mM disaccharide. Subsequent procedures were performed as already described.

Trimethylsilyl derivatives of oxidation products. — After the addition of catalase, the mixtures were reduced with sodium borohydride, and freeze-dried, and the products acetylated with acetic anhydride (0.5 mL)–anhydrous pyridine (0.5 mL) for 120 min at 80°. Then, the acetylation products were extracted with $CHCl_3$, and the extracts were combined, washed twice with distilled water, dried (sodium sulfate), and evaporated to dryness. The products were dissolved in 3 mL of methanol and incubated with 1 mL of sodium methoxide solution (0.5 g in 100 mL of methanol) for 20 min at room temperature. Then, the mixtures were treated with Amberlite IR-120 (H^+) cation-exchange resin to adjust the pH to neutral, and, after filtration, evaporated to dryness under diminished pressure and dried *in vacuo*. The deacetylated sugars were incubated with anhydrous pyridine (0.3 mL), hexamethyldisilazane (0.2 mL), and chlorotrimethylsilane (0.1 mL) for 30 min at 80°. Then, 2 μL of each mixture was subjected to g.l.c. and g.l.c.–m.s. The procedures mentioned were performed quantitatively.

G.l.c. — A Shimadzu GC-9A gas chromatograph equipped with a flame-ionization detector was used. Analysis was carried out by using a glass column (3.0 mm i.d. × 2 m) packed with 5% of silicone SE-52 on Chromosorb W(AW) (80–100 mesh) for trimethylsilyl derivatives. The temperature rises were programmed at a rate of 5°/min from 180 to 280°, while the nitrogen flow-rate was 40 mL/min and the temperature of the detector oven was 300°. Areas of the gas chromatographic peaks of oxidized products were calculated by use of a Shimadzu Chromatopac integrator C-R3A.

G.l.c.-m.s. — A JEOL Model JMS-D100 mass spectrometer connected to a JEOL Model JGC-20K gas chromatograph equipped with a glass column (2.0 mm i.d. × 1.0 m) packed with 5% of Silicone OV-1 on Chromosorb W (100–120 mesh was used. The m.s. conditions were as follows: the temperature rises were the same as for g.l.c.; gas, helium (0.65 kg/cm^2); ionizing voltage, 28 eV; accelerating voltage, 3000 V; and ion-source temperature, 250°.

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