Phosphorylated Glycans Produced from Nonreducing Mono- and Oligosaccharides by the Action of P_2O_5 in Dimethyl Sulfoxide, and Their Interactions with Concanavalin A[†]

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The action of P_2O_5 in DMSO on methyl α -D-glucopyranoside, sucrose and trehalose afforded nondialyzable, phosphorylated glycans in 6~34% yields. Polysucrose has a molecular weight of ~9,500. The synthetic glycans consist of carbohydrate (46~59%) and P (11.4~13.1%) and show reducing sugar values (5.0~30.8%). The alkaline hydrolysis of polysucrose was accompanied with a depolymerization and afforded sugar phosphates and oligosaccharides. The periodate oxidation gave formic acid (0.15~0.34 mole) and formaldehyde (0.07~0.17 mole/monosaccharide residue). The methylation study indicated their variously branched structures. 2,3,4,6-Tetra-O-methyl-D-glucose was found in only 0.7~3.2% yields, and this is in agreement with their weak precipitation reactions with Con A. It is considered that the glycans are produced from nonreducing mono- and oligosaccharides by dehydration, transglycosidation and esterification with phosphate.

The action of P_2O_5 ,^{1,2)} H^+ or $H^+-P_2O_5$,^{3a,3b)} on reducing mono- and oligosaccharides in DMSO affords glycans. The synthetic glycans produced by the P_2O_5 -DMSO method¹⁾ contain P originated from P_2O_5 and those prepared by the H^+ -DMSO method^{3a,3b)} contain -O-CH₂-O- originated from DMSO. However, the polymerization of nonreducing mono- and oligosaccharides by these methods has not been reported.

The present paper describes novel, phosphorylated glycans prepared by the P_2O_5 -DMSO method¹⁾ from methyl α -D-glucopyranoside, sucrose and trehalose, their proposed structure on the basis of methylation, alkaline

hydrolysis and periodate oxidation, and their weak precipitation reactions with Con A.

RESULTS AND DISCUSSION

The action of P_2O_5 in DMSO on methyl α -D-glucopyranoside, sucrose and trehalose afforded the corresponding nondialyzable glycans in 6~34% yields. As shown in Table I, the glycans consist of carbohydrate (46~59%) and P (11~13%) which corresponds to 33~42% of PO_4^{3-} . All the glycans show IR absorptions at 3300~3500 (OH), ~1240 (P=O) and 1040~1100 cm^{-1} (C-O-C). Their specific rotations indicate the presence of both α - and β -D-configurations in these polymers. In every sample, the initial monosaccharides were detected in the total hydrolyzate (3 N HCl, 100°C, 18 hr) by paper chromatography. The molecular weight of polysucrose for the high molecular weight portion (tube number 20~30, see the experimental) was calculated as ~9,500 on the basis of $s_{20,w}=1.11$ by assuming the specific volume: 0.613.

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The following abbreviations are used: G-1-P, α -D-glucose-1-phosphate; Con A, Concanavalin A; GLC, gas-liquid chromatography; V_o , void volume; V_s , salt-region volume.

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TABLE I. ANALYSIS OF SYNTHETIC GLYCANS PRODUCED FROM NONREDUCING MONO- AND OLIGOSACCHARIDES

Glycan	Yield (%)	Hexose (%)	Phosphorus ^{b)} (%)	Reducing sugar value (%)	$[\alpha]_D^{25}$ (degree) (water)
Poly methyl α -D-glucopyranoside	6	59.0	12.0	5.0	+20 ($c=0.5$)
Polytrehalose	16	50.0	13.1	25.5	+35 ($c=0.76$)
Polysucrose	34	46.3 ^{a)}	11.4	30.8	+ 7.6 ($c=0.92$)

^{a)} A paper chromatographic examination (1-butanol-acetic acid-water, 4:1:5, v/v) indicated the presence of D-glucose ($R_f=0.15$) and D-fructose ($R_f=0.21$).

^{b)} Ash (40~50%) involves in the glycans, and this is considered to exist as metallic cation salts which have been formed in the course of the preparation.

FIG. 1. Comparison of the Elution Pattern of Alkali-treated Polysucrose (x---x) with that of the Untreated Polysucrose (●—●) on a Column (1.7 × 220 mm) of Bio-Gel P-2.

The column was eluted with 10% ethanol and fractions of 7.5 g each were collected. A screening test was carried out by the anthrone method. See the text for the experimental details.

Alkaline degradation

The alkaline hydrolysis of polysucrose, by which 3'-mononucleotides are produced from nucleic acids,⁴⁾ afforded oligosaccharides and sugar phosphates. This is accompanied with a depolymerization. The depolymerization is evident by its elution pattern in comparison with the initial sample on a column of Bio-

Gel P-2 as shown in Fig. 1. Both the alkali-labile glycoside and phosphate ester are considered to be cleaved by the alkaline treatment. Structural study of the products was not carried out, but one component ($R_{\text{sucrose}}=0.68$) showed an identical mobility with that of G-1-P by a paper chromatographic examination.

Methylation

Permethylation was carried out by repeating Hakomori's method⁶⁾ and was confirmed by almost complete disappearance of IR absorption at $3000\sim3500\text{ cm}^{-1}$ for free OH. The permethylated products were isolated in 31~80% yields. However, their considerable degradation was found in the repeated methylations. This is because of the alkaline property of reaction medium, and the alkaline degradation is considered to occur as described above. As shown in Table II, the GLC analysis of the hydrolyzed products indicates the presence of mono-, di-, tri- and tetra-*O*-methyl-D-glucoses (72.6~90.4%) and free D-glucose (9.6~27.4%). This indicates variously branched structures for the glycans. 2,3,4,6-Tetra-*O*-methyl-D-glucose had been isolated in ~40% yield from the acid hydrolyzate of the permethylated poly D-glucose in our previous work.^{1c)} However, this compound was detected in only 0.7~3.2% yields by GLC in the present study because the depolymerized products were dialyzed out in the present methylation.

Splitting *O*-methyl signal appears at δ 3.44,

TABLE II. GLC DATA FOR O-METHYLATED-D-GLUCOSES FROM THE ACID HYDROLYZATES OF PERMETHYLATED SYNTHETIC GLYCANS (%)^{a)}

O-Methyl-D-glucose	Polytrehalose	Polysucrose	Polycellobiose	Polylactose	Polymaltose
2,3,4,6-	3.2	0.7	1.6	0.9	1.2
2,3,4-	3.4	— ^{b)}	2.2	—	5.9
2,3,6-	12.1	—	4.6	—	4.3
2,6;4,6;3,6-	13.6	—	7.1	—	10.0
2,3;2,4;3,4-	13.9	—	15.2	—	10.1
6-	8.1	—	11.8	—	5.6
3-	7.8	—	5.3	—	5.6
2-	12.5	—	12.6	—	6.0
2,4,6;3,4,6;4-	15.9	—	12.2	—	33.7
Free D-glucose	9.6	—	27.4	—	17.6

^{a)} Analyzed as both methylglucosides and alditol acetates.^{b)} Not determined.

3.48, 3.51 and 3.55 ppm in the NMR spectrum of poly methyl α -D-glucoside. This also supports the substituted structures of D-glucopyranosyl residue in the polymer.

Periodate oxidation

Proton signals of HCOOD at δ 8.20~8.28 ppm and H₂C=O at δ 4.82~4.90 ppm appear in the NMR spectra of the periodate oxidation products (Table III), and the pro-

TABLE III. HCOOH AND H₂C=O PER MONO-SACCHARIDE RESIDUE FROM THE PERIODATE OXIDATION OF SOME SYNTHETIC GLYCANS IN D₂O AS EXAMINED BY NMR SPECTROSCOPY (MOLE)

Glycan	HCOOH ^{a)}	H ₂ C=O ^{b)}
Poly D-glucose	0.34	0.10
Polytrehalose	0.15	0.08
Polysucrose	0.16	0.14
Polycellobiose	0.18	0.07
Polylactose	0.25	0.17
Polymaltose	0.15	0.11
Rabbit-liver glycogen	0.17	0.00
Yeast mannan	0.04	0.00

^{a)} Appeared at δ 8.20~8.28 ppm.^{b)} Appeared at δ 4.82~4.90 ppm.

duction of H₂C=O was in the amounts of 0.07~0.17 mole per monosaccharide residue. The H₂C=O proton is considered to originate from reducing end group, and the result is in agreement with the reducing sugar values observed. Reducing groups newly formed by

the oxidation reaction is disregarded in the present conditions.⁶⁾ The presence of furanosyl structure was not examined in the present study.

Interactions with Con A

The best condition for the precipitation reactions with Con A was chosen by changing both the amounts of poly D-glucose and Con A in various reaction periods. As shown in Table IV, the extents of the precipitation re-

TABLE IV. PRECIPITATION REACTIONS WITH CONCAVALIN A

Glycan	Extent of the precipitation ^{a)}
Poly D-glucose	0.21
Polytrehalose	0.03
Polysucrose	0.03
Polycellobiose	0.03
Polylactose	0.04
Polymaltose	0.03
Poly D-mannose ^{b)}	0.01
Yeast mannan	2.18
Rabbit-liver glycogen	1.00

^{a)} The extent of precipitation is compared with that of rabbit-liver glycogen (1.00).^{b)} This was prepared from D-mannose by the P₂O₅-DMSO method; hexose, 33.6%.

actions for the synthetic glycans are weaker than that of rabbit-liver glycogen. Poly D-glucose shows a relatively high value. The data indicate that the synthetic glycans have

only a small amount of α -D-glucopyranosyl residue having free OH at C₃, C₄ and C₆.^{7a,7b)} This is in agreement with the methylation data. All the synthetic glycans did not inhibit the precipitation reaction of yeast mannan with Con A. The results excludes the solubilization of the glycans by making their complexes with Con A. No precipitation was detected in the reaction of poly *N*-acetyl-D-glucosamine with the wheat germ agglutinin.^{6c)}

These data concludes that the polymerization involves dehydration, transglycosidation and esterification with phosphate. Not only mono- but also di- and triphosphate esters may exist between nonhemiacetal or non-hemiketal OH groups of sugar residues (*cf.* nucleic acids), and the unsubstituted hemiacetal and hemiketal OH groups remain in considerable amounts on the polymers. This is in good agreement with the fact that the products have relatively high molecular weight and high reducing sugar values. The transglycosidation involved is supported by the fact that 2,3,4,6-tetra-*O*-methyl-D-glucose was detected by GLC analysis in the acid hydrolyzate of permethylated polylactose, and D-glucitol was detected in the acid hydrolyzate of polytrehalose which had been reduced with NaBH₄. However, it is evident that G-1-P is not an intermediate for the polymerization because no polymers were detected by the present conditions from G-1-P, adenosine and uridine. Alkali-labile glycoside⁸⁾ and phosphate ester⁴⁾ are cleaved by alkali to afford neutral oligosaccharides and sugar phosphates.

EXPERIMENTAL

General methods. NMR spectra were recorded at 60 MHz with a Hitachi spectrometer (R-24) on solutions in D₂O with sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionate as internal standard. IR spectra were recorded with a Hitachi grating spectrometer (215) (KBr). Specific rotations were recorded in aqueous solutions using a cell with a light path of 1.0 cm on a Yanagimoto automatic polarimeter (OR-50).

GLC analysis was performed with a Shimadzu gas-liquid chromatograph (GC-5A) with a hydrogen flame

ionization detector. A glass column (4 mm $\times$ 2 m) packed with 15% polybutandiol glycol succinate on Celite 545 was used for the analysis of methyl glycosides at 175°C and that packed with 0.5% ethylene glycol adipate on Chromosorb W (60~80 mesh) was used for the analysis of alditol acetates at 180°C.

The periodate oxidation was performed on D₂O solutions involving 5~10 mg of sample in an NMR sample tube (d. 0.4 mm) at 25°C in the dark for 72 hr.⁹⁾

The permethylation was performed by Hakomori's method.⁵⁾ After the reaction was over, the excess CH₃I was evaporated out from the reaction mixture, and the residue was dissolved in a small volume of distilled water and dialyzed against running water and then in distilled water overnight. The nondialyzable part was concentrated and lyophilized. The methylation was repeated until the IR absorption at 3000~3500 cm⁻¹ for free OH disappeared almost completely. Its complete methanolysis was carried out in methanol containing 5% HCl by refluxing for 10 hr. A part of the methylated product was analyzed by GLC analysis. The other part was converted into alditol acetates by the conventional method and the GLC analysis was performed.

Hexoses were analyzed by the phenol-H₂SO₄ method¹⁰⁾ and reducing sugar values by the ferricyanide method¹¹⁾ with use of D-glucose as a standard.

Paper chromatography was carried out by descending method with 1-butanol-acetic acid-water-ethanol (50: 12: 25: 5, v/v).

Materials. The synthetic glycans used in the present study were prepared by the P₂O₅-DMSO method as reported previously.^{1c)} Con A is a product of the Pharmacia Fine Chemicals, Sweden. Wheat germ agglutinin was isolated in our laboratory from wheat germs by an affinity chromatography on a chitin column.¹²⁾

Polymerization. To 20 ml of anhydrous DMSO was added ~2.0 g of P₂O₅ in small portions with stirring in an ice bath to afford a homogeneous solution. To the solution was added 1.0 g of mono- and oligosaccharides to be polymerized, and the mixture was kept below 10°C for a few hr and kept at 37°C with occasional stirring for 3 days. The reaction mixture was poured into a large volume (~300 ml) of acetone, and the mixture was stirred and kept at room temperature overnight to afford an amorphous or syrupy product. The supernatant solution was decanted and the residue was dissolved in ~20 ml of distilled water. The solution was dialyzed against running water through a cellophane tubing for 2 days. The nondialyzable portion was concentrated *in vacuo* at temperature below 45°C into a small volume. This was lyophilized to afford an amorphous product.

The polymerized products were dissolved in a small

volume of water at room temperature, and insoluble material was centrifuged out. The supernatant solution was applied to a column (1.7×222 cm) of Bio-Gel P-2 and the column was eluted with 10% ethanol. Fractions of 7.5 g each were collected (V_0 =tube number 23, V_s =tube number 59). A screening test was carried out by the phenol- H_2SO_4 method.¹⁰⁾ Only the fraction of tube number 20~30 was used in the present study and the other fractions (tube number 31~65) were not examined.

Alkaline degradation. Polysucrose (0.8 g) was dissolved in 100 ml of 0.3 N NaOH and the solution was kept at 37°C for 18 hr. The reaction products were diluted to ~200 ml with distilled water. The solution was applied to a column (5×12 cm) of IRA 120 (H^+) and then the eluate was applied to a column (3×45 cm) of IRA 410 (OH^-). Both the columns were washed with ~1 liter of distilled water. a) The eluate and the washing were combined and concentrated *in vacuo* into a syrup. To the syrup was added ~100 ml of 95% ethanol to afford an amorphous precipitate; yield 0.45 g (56%), $[\alpha]_D^{20} + 1.0^\circ$ ($c=1.0$, water), hexose 21.7%, reducing sugar value 8.3%. IR: ν_{max}^{KBr} ~3400 (OH), 1240 (P=O), 1020~1040 (C-O-C). b) The column of IRA 410 was eluted with 5% KOH, and fractions of 15 g each were collected. Fractions which showed a positive anthrone reaction were combined and 3 volumes of ethanol were added to afford a syrup. A paper chromatographic examination indicated the presence of at least three components ($R_{sucrose}=0.51, 0.68$ and 0.86) in the syrup. The isolation and structural study of the compounds are now in progress.

Precipitation reactions with Con A. The assay was carried out at room temperature for exactly 3 hr in the following mixture (total 3.0 ml): a) Con A (0.7 g) dissolved in 1.0 ml of 1 M NaCl, b) a synthetic glycan solution (1.0 ml) involving 0.5 mg of D-glucose content as assayed by the phenol- H_2SO_4 method, c) 0.5 ml of 4 M NaCl, and d) 0.5 ml of 0.1 M phosphate buffer solution (pH 7.3). The extent of the turbidity produced was determined by the absorption at 420 nm and was compared with that of rabbit-liver glycogen (1.00).¹³⁾

Inhibition tests on the precipitation of yeast mannan with Con A. Con A (1.0 mg) was dissolved in 1.0 ml of 1 M NaCl, and to the solution was added 0.5 ml of a synthetic glycan solution involving 0.5 mg of D-glucose content. To the mixture was added 0.5 ml each of 4 M NaCl solution, 0.1 M phosphate buffer solution (pH 7.3) and yeast mannan solution (0.5 mg/

0.5 ml). The mixture was incubated at room temperature for exactly 1 hr, and the extent of the turbidity produced was analyzed at 420 nm against a blank.

Precipitation reaction with wheat germ agglutinin. Wheat germ agglutinin (354 μ g) was dissolved in 2.0 ml of 1 M NaCl, and to the solution was added 0.5 ml of a solution of poly N-acetyl-D-glucosamine (1.0 mg/0.5 ml). To the mixture was added 0.5 ml of 0.1 M phosphate buffer solution (pH 7.5) containing 4 M NaCl. The mixture was incubated at room temperature for exactly 24 hr and the turbidity produced was analyzed at 420 nm against a blank.

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