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Extracellular Polysaccharide of *Cladosporium herbarum*[1] Studies on Fungal Polysaccharide. XIII[2]

Toshio Miyazaki[3a] and Yasuta Naoi[3b]

Tokyo College of Pharmacy[3a] and Tokyo Metropolitan Research Laboratory of Public Health[3b]

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Major water-soluble extracellular polysaccharide of *C. herbarum* obtained by DEAE-cellulose column fractionation and zone electrophoresis using 1% $Na_2B_4O_7$ of crude polysaccharide is a galactomannan, $[\alpha]_D^{25}$ +27° (c=1.0, H_2O), which was composed of D-galactose and D-mannose=1.0: 1.5. From the results of periodate oxidation, Smith-type degradation, and methylation studies showed that the polysaccharide has a highly branched structure and contains 1→2 main linkage of mannopyranose with 1→4 linked galactopyranose, and the glycan was branching at C4 position of mannose residue. The terminal residues are galactofranose and mannopyranose.

Cladosporium herbarum, a kind of "black yeast" belongs to fungi imperfecti widely distributed in nature. Some species belonging to the genus *Cladosporium* are well known as pathogenic fungi which cause chromoblastmycosis and as contaminating fungi on foods.

Recently, Lloyd[4] reported the presence of an intracellular peptide-phosphogalactomannan complex in the yeast form of *C. werneckii* and the complex consisted of 78% mannose, 13% galactose, 9.6% protein, and 3.2% phosphate. However, extracellular polysaccharides of genus *Cladosporium* have not been examined in detail, particularly in the filamentous form of *Cladosporium* cultured in liquid Sabouraud medium.

Cladosporium herbarum is a dimorphic fungus, and colonies are typically slow-glowing, velvety, black, and often yeast-like. Therefore, this is called "black yeast". The extracellular polysaccharide of *C. herbarum* isolated from the culture liquid was treated with pronase and by the Sevag method,[5] followed by repeated DEAE-cellulose column chromatography using sodium hydrogen carbonate and then sodium borate for elution. The major fraction, F-I, was further purified by zone electrophoresis using glass powder and borate buffer.

PF-I, the purified major polysaccharide thus obtained showed $[\alpha]_D^{25}$ +27° (c=1.0, H_2O), contained no phosphorus and 96% of total hexose (by phenol-sulfuric acid method[6]) and was electrophoretically pure. The component sugars of this polysaccharide were identified as D-galactose, D-mannose, and a trace of glucose by paper chromatography of the hydrolysate, and the molar ratio of galactose to mannose was estimated approximately as 1.0: 1.5 by the procedure of Dubois, *et al.*[6]

On periodate oxidation, the polysaccharide consumed 1.45 mole of periodate per anhydrohexose unit (extrapolated to zero time from the periodate consumption curve), the value of formic acid liberated from the unit was 0.24 mole, and that of formaldehyde was 0.15 mole after 72 hr.

1) A part of this work was presented at the 88th and 89th Annual Meetings of the Pharmaceutical Society of Japan, Tokyo, April 1968 and Nagoya, April 1969.
2) Part XII: T. Miyazaki and N. Oikawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 2545 (1973).
3) Location: a) *Kitashinjuku 3-chome, Shinjuku-ku, Tokyo, 160, Japan*; b) *24-1, Hyakunin-cho 3-chome, Shinjuku-ku, Tokyo, 160, Japan.*
4) K.O. Lloyd, *Biochemistry*, **9**, 3446 (1970); *idem*, *FEBS Letters*, **11**, 91 (1970).
5) M.G. Sevag, *Biochem. Z.*, **273**, 419 (1934).
6) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

The periodate-oxidized polysaccharide was treated with sodium borohydride followed by mild acid hydrolysis (Smith-type degradation). Paper chromatographic analysis of the hydrolysate revealed the presence of galactose and mannose in addition to threitol and glycerol. The molar ratio, approximately 1.0: 7.2: 7.0: 4.4 (Gal–Man–Thr–Gly), was estimated by the procedure of Dubois, *et al.*[6] for hexoses and of Lambert and Neish[7] for polyalcohols after the hydrolysate was separated by paper chromatography.

Acid hydrolysis of the polysaccharide using 0.01N sulfuric acid for 4 hr at 100°, gave D-galactose corresponding to 13.8% of total hexose. Non-dialysable fragment by this treatment contained D-galactose and D-mannose in the ratio of 1.0: 2.3. When the non-dialysable fragment was hydrolysed with 0.1N sulfuric acid for 3 hr at 100°, galactose and mannose in the ratio 4.4: 1.0 were formed. When the non-dialysable fragment was hydrolysed with 0.5N sulfuric acid for 2 hr at 100°, galactose and mannose were liberated in the ratio of 1.0: 2.1, and the final hydrolysis of the non-dialysable fragment using 2N sulfuric acid for 6 hr at 100° gave only mannose.

The results of partial hydrolysis study suggested some structural features of PF-I that this galactomannan has an acid-resistant core consisting of mannose and branching moieties involving acid-labile galactofuranose.

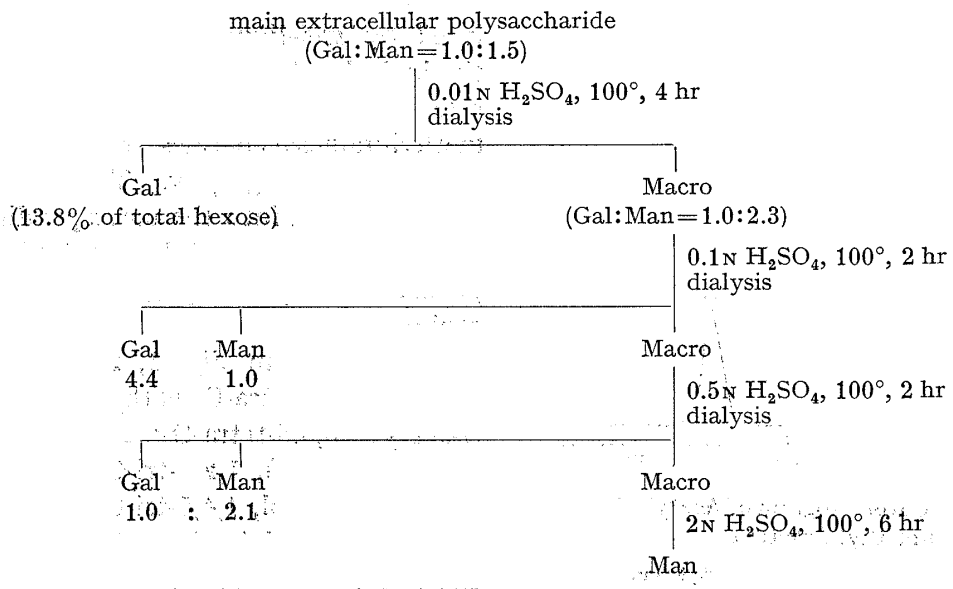


Chart 1. Partial Acid Hydrolysis of the Polysaccharide

Macro: non-dialyzable material

After complete methylation of the polysaccharide using the methods of Hakomori[8] and Purdie,[9] followed by hydrolysis with formic and sulfuric acids, the methylated monosaccharides formed were examined by paper chromatography, thin-layer chromatography, and by paper electrophoresis. From the results of these chromatographic analyses, the molar ratio of di-O-methyl-, tri-O-methyl-, and tetra-O-methyl- monosaccharides was determined as 1: 2: 1.

Di-O-methyl-monosaccharide fraction separated by paper electrophoresis using 1% borate was identified as 3,6-di-O-methyl-monosaccharide (MG value, 0.38). Tri-O-methyl-monosaccharide fraction of the paper electrophoresis using 1% $Na_2B_4O_7$ was identified as 3,4,6-tri-O-methyl- and 2,3,6-tri-O-methyl-monosaccharide (MG value, 0.25 and 0.00).

7) M. Lambert and A.C. Neish, *Can. J. Research.*, **28B**, 83 (1956).
8) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
9) T. Purdie and L.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).

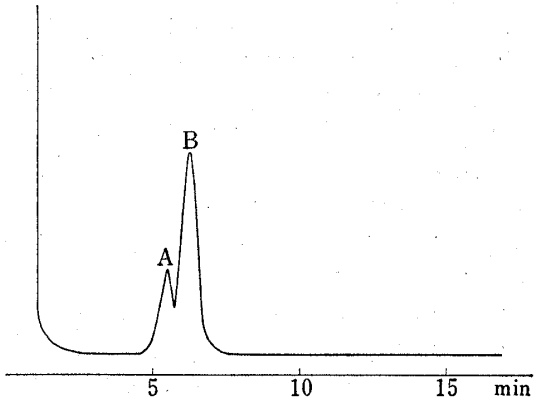


Fig. 1. Gas Chromatogram of Acetyl Alditol of the Tetra-O-methyl Fraction

A: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol
B: 1,4-di-O-acetyl-2,3,5,6-tetra-O-methylgalactitol

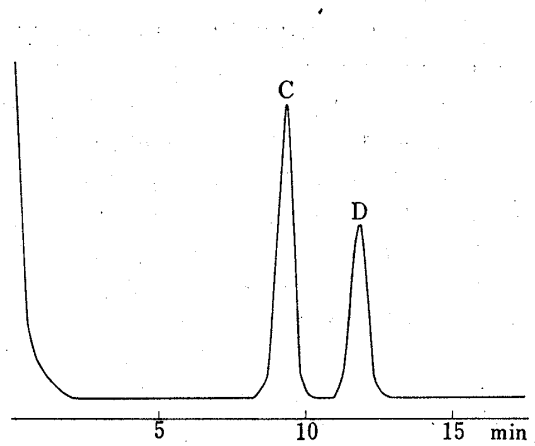


Fig. 2. Gas Chromatogram of Acetyl Alditol of the Tri-O-methyl Fraction

C: 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylmannitol
D: 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol

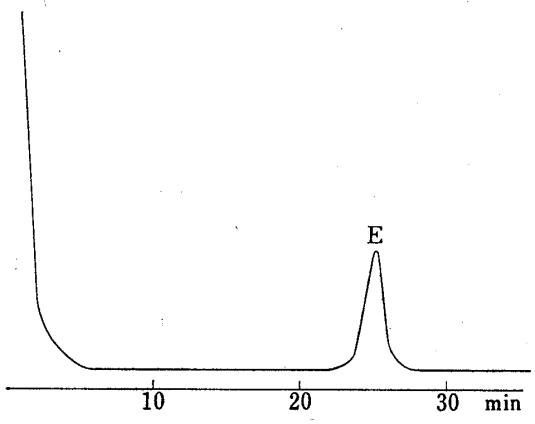


Fig. 3. Gas Chromatogram of Acetyl Alditol of the Di-O-methyl Fraction

E: 1,2,4,5-tetra-O-acetyl-3,6-di-O-methylmannitol

O-Methyl-monosaccharides were also analyzed by gas–liquid chromatography. From the hydrolysate of the methylated polysaccharide, di-O-methyl-, tri-O-methyl-, and tetra-O-methyl-monosaccharides were separated by paper chromatography. Then each fraction was converted into the corresponding methyl-glycosides and alditol acetates,[10] and the products analyzed by gas–liquid chromatography.

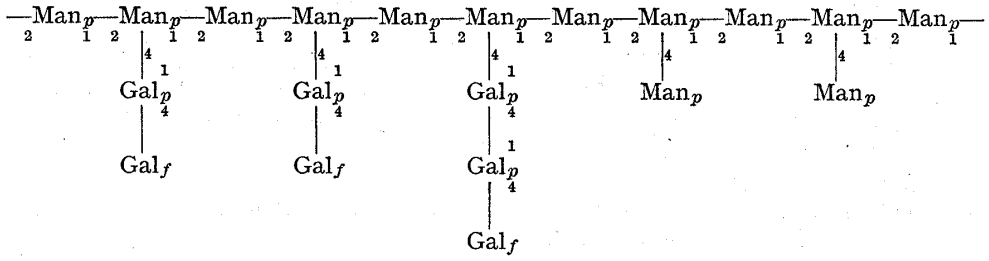
As shown in Fig. 1, 2, and 3, 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol, 1,4-di-O-acetyl-2,3,5,6-tetra-O-methylgalactitol(molar ratio, *ca.* 1: 1.5), 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-mannitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol (molar ratio, *ca.* 1.4: 1), and 1,2,4,5-tetra-O-acetyl-3,6-di-O-methyl-mannitol were detected from the tetra-O-methyl, tri-O-methyl, and di-O-methyl fractions.

Table I. Relative Retention Times of Acetyl Alditols of the Methylated PF-I

PF-I component		Authentic O-methylalditol
1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol	1.00	1.00
1,4-Di-O-acetyl-2,3,5,6-tetra-O-methyl galactitol	1.15	1.15
1,2,5-Tri-O-acetyl-3,4,6-tri-O-methyl mannitol	1.85	1.85
1,4,5-O-Acetyl-2,3,6-tri-O-methyl galactitol	2.32	2.32
1,2,4,5-Tetra-O-acetyl-3,6-di-O-methyl mannitol	4.68	——

10) B. Haken, L. Bengt, and S. Sigfrid, *Acta. Chem. Scand.*, **21**, 1801 (1967).

The results of alditol acetate method was quite consistent with that of the methylglycoside method. From these results, it was concluded that this polysaccharide has a highly branched structure and a probable structure of the main portion in the polysaccharide will be as follows:



Peptide-phosphogalactomannan from the cell wall of *C. werneckii* is a complex which contains both acid-sensitive and alkali-sensitive linkages, and it also contains 1—2 linked mannoses with smaller amounts of 1—6 and 1—3 linked mannose residues, and further all of the galactose residues present as terminal groups. In the case of PF-I, as described above, it has a highly branched structure which contains 1—2 linked mannan core and 1—4 linked galactopyranose residues with terminal galactofuranose and mannopyranose residues. Therefore, it is certain that they are evidently different from each other. Polysaccharide component from the cell wall of *C. herbarum* will be reported in a following paper.

Experimental

Isolation of Crude Extracellular Polysaccharide——The organism used in this study, *Cladosporium herbarum* 4000, was kindly supplied by the National Institute of Hygienic Sciences, Tokyo.

Incubation was carried out at 25° for 40 days in an Erlenmeyer flask of 300 ml capacity containing 150 ml of Sabouraud liquid medium containing 4% glucose and 1% dialyzable peptone.

After the mycelium was removed by filtration, the culture fluid (10 liters) was dialzyed in a Visking cellulose tubing against running water for 3 days to remove excess medium constituents and low-molecular products. Non-dialyzable solution was concentrated to a small volume under a reduced pressure below 40°. A precipitate formed by the addition of 4 volumes of ethanol, and it was collected by centrifugation, washed with EtOH, acetone, and dried *in vacuo*.

Digestion of Crude Polysaccharide with Pronase——About 10 g of the crude polysaccharide was dissolved in 100 ml of distilled water and adjusted pH to 7.8 with Na_2CO_3. To the solution was added pronase (0.5 g) and incubated for 4 days at 30°. Then the mixture was dialyzed in a Visking cellulose tubing against running water for 2 days. The solution in the tube was concentrated to 100 ml and shaken vigorously for 0.5 hr with 20 ml of $CHCl_3$–BuOH (4: 1). The mixture was centrifuged to collect the aqueous layer, and the same procedure was repeated until a gelatinous substance was no longer formed in the mixture. After centrifugation, the supernatant was concentrated to a small volume under a reduced pressure below 40°, and 4 volumes of EtOH containing 0.1% AcOK was added to this concentrate. The precipitate was collected by centrifugation, washed with EtOH, acetone, and ether, and dried *in vacuo*. The same procedure was repeated 3 times. Yield, 0.3 g per 1 of cultivated fluid.

Fractionation of Crude Polysaccharide by Ion-Exchange Chromatography——The crude polysaccharide (1.28 g) was applied to a DEAE-cellulose column (OH^-), (4.5×50 cm). Stepwise elution was carried out with H_2O, 0.01M, 0.05M, 0.1M $NaHCO_3$, and 0.1N NaOH at the flow rate of 100 ml/hr. Each fraction (0.1 ml) was added with 2 ml of H_2O and 4 ml of 0.2% anthrone reagent, and the optical density was read at 625 mμ on colorimeter. Each fraction was dialyzed in a Visking cellulose tubing against distilled water for 4 days. The internal solution of the tubes was concentrated to a small volume *in vacuo* and 10 volumes of EtOH was added to the concentrate. The precipitate formed was collected by centrifugation, washed with EtOH, acetone, and ether, and then dried *in vacuo*. Yield was as follows: H_2O eluate, 584 mg (45.6%); 0.01M $NaHCO_3$ eluate, 132 mg (10.3%), 0.05M $NaHCO_3$ eluate, 382 mg (29.8%), 0.1M $NaHCO_3$ eluate 158 mg (12.3%); 0.1N NaOH eluate, trace.

H_2O fraction (945 mg) in H_2O (30 ml) was further submitted to a column (3.5×45 cm) of DEAE-cellulose. Stepwise elution with H_2O, 0.01M, 0.05M, 0.1M $Na_2B_4O_7$, and finally with 0.1N NaOH was carried out in the same way as described above. Yields were as follows: H_2O eluate (F-I) 655 mg (69.3%); 0.01M $Na_2B_4O_7$ eluate (F-2) 72 mg (7.6%); 0.05M and 0.1M $Na_2B_4O_7$ eluate, trace; 0.1N NaOH eluate, nil.

Purification of F-I by Zone Electrophoresis——Zone electrophoresis of F-I (100 mg) was carried out using glass powder as a supporting medium (1×10×40 cm), for 6 hr in 1% $Na_2B_4O_7$ under as applied current of 35 mA. After migration, the zone was cut into 40 segments, each segment was quantitatively extracted

with a constant volume of water and was determined with anthrone reagent. Yield of the main peak (PF-I) was about 80% and the minor peaks was about 8%.

Component Sugars of the Major Fraction PF-I——PF-I (20 mg) in 3 ml of 1N H_2SO_4 sealed in a tube was heated in a boiling water bath for 6 hr. After neutralization ($BaCO_3$) and filtration, a portion of the hydrolysate was concentrated and applied to Whatman No. 1 filter paper for detection of component sugars. Paper chromatography was carried out by the ascending method, using AcOEt–pyridine–H_2O (10: 4: 3). Sugars were detected on the paper chromatogram by spraying a solution of *p*-anisidine hydrochloride and alkaline $AgNO_3$. PF-I showed the presence of galactose, mannose and trace of glucose, molar ratio 1.0: 1.5.

Properties of PF-I——Paper electrophoresis of PF-I using 0.05M borate buffer (pH 10.0) showed a single spot (detected with the periodate-Schiff reagent[11]). PF-I, $[\alpha]_D^{25}$ +27° (c=1.0, H_2O), contained 96% hexose (by phenol–H_2SO_4) and no phosphate.

Periodate Oxidation of PF-I——PF-I (26 mg) was oxidized in 50 ml of 0.018 mole $NaIO_4$ at room temperature in the dark. A blank solution containing no glycan was processed similarly. An aliquot of 3 ml was taken at different periods for the determination of $NaIO_4$ consumption and formation of HCOOH and HCHO by the procedures of Maraprade,[12] Whistler,[13] and of O'Dea and Gibbons,[14] respectively. The number moles of $NaIO_4$ consumed per anhydro hexose unit of polysaccharide was as follows: 0.53 (1 hr), 0.88 (3 hr), 0.99 (12 hr), 1.14 (24 hr), 1.28 (48 hr), 1.45 (72 hr), 1.61 (96 hr). The value of HCOOH: 0.03 (3 hr), 0.12 (12 hr), 0.16 (24 hr), 0.21 (48 hr), 0.24 (72 hr), 0.27 (96 hr); value for HCHO was 0.10 (2 hr), 0.12 (12 hr), 0.13 (24 hr), 0.15 (72 hr), 0.15 (96 hr).

Smith-type Degradation of Periodate-Oxidized PF-I——After oxidation of PF-I as described above, excess $NaIO_4$ was decomposed by the addition of 0.4 ml of ethylene glycol. The reaction mixture was dialyzed in a Visking cellulose tubing against distilled water with stirring for 18 hr, the internal solution was concentrated to a small volume *in vacuo*, and the resultant polyaldehyde was reduced by stirring with 100 mg of $NaBH_4$ at room temperature for 17 hr. Excess of $NaBH_4$ was decomposed with AcOH. The reaction mixture was dialyzed in a Visking cellulose tubing against distilled water, the internal solution was evaporated to dryness, and the residue was heated with 1N H_2SO_4 (2 ml) in a boiling water bath for 4 hr. The hydrolysate was neutralized with $BaCO_3$, $BaSO_4$ formed was removed by filtration, and the filtrate was concentrated to a small volume *in vacuo* to be examined by paper chromatography using AcOEt–pyridine–H_2O (10: 4: 3). Four spots corresponding to standard galactose, mannose, threitol and glycerol were detected as the main products on the paper chromatogram. In order to estimate the relative molar ratio, the syrup was spotted on a filter paper and multiple developing was carried out by the ascending method, using AcOEt–pyridine–H_2O (10: 4: 3). After air drying, the corresponding area on the paper chromatogram were quantitatively extracted with constant volume of distilled water and the extract filtered through a sintered glass filter.

Hexoses were determined by the method of Dubois, *et al.*,[6] and threitol and glycerol by the method of Lambert–Neish.[7] The molar ratio was approximately 1.0: 7.2: 7.0: 4.4 (Gal–Man–Thr–Gly).

Partial Acid Hydrolysis of PF-I——PF-I (45 mg) was heated with 0.01N H_2SO_4 (5 ml) at 100° for 4 hr and followed by dialysis, and then the external solution (600 ml) of the hydrolysate was evaporated to about 20 ml *in vacuo*. After neutralization with $BaCO_3$ and filtration, the filtrate was concentrated to a syrup. Paper chromatographic analysis of the syrup using AcOEt–pyridine–H_2O (10: 4: 3) revealed the presence of galactose (5.95 mg). The internal solution was concentrated to dryness (38.7 mg), and then heated with 0.1N H_2SO_4 (10 ml) at 100°, 3 hr. The dialyzable fragment (13 mg) from the hydrolysate, which was treated with the same procedure as described above, gave two components, galactose and mannose (molar ratio, 4.4: 1.0). The internal solution was heated with 0.5N H_2SO_4 (10 ml) in a boiling water bath for 2 hr and then dialyzed. The external solution was concentrated to a small volume, neutralized with $BaCO_3$, and filtered. The filtrate (free from barium ions by Amberlite IR-120(H^+)) was evaporated to a syrup. The residue was treated with the same procedure as described above, to give two components (11.6 mg), galactose and mannose (molar ratio, 1.0: 2.1). The yielding non-dialyzable material was further heated with 2N H_2SO_4 (10 ml) at 100°, for 6 hr, the hydrolysate gave only one component, mannose.

Methylation of PF-I——NaH (1.5 g) was mixed with 15 ml of $(CH_3)_2SO$ and the mixture was heated 60° for 1 hr with stirring. To this solution was added a solution of PF-I (50 mg) in 5 ml of $(CH_3)_2SO$ with stirring. After 5 hr, 0.4 ml of CH_3I was added dropwise to the reaction mixture with stirring at room temperature and the mixture was stirred for 12 hr. All the procedure were carried out in nitrogen atmosphere. The methylation product thus obtained was methylated again under the above condition. The partially methylated PF-I thus obtained was dissolved in CH_3I (5 ml) and then stirred in a water bath of 40° for 12 hr with occasional addition of Ag_2O (50 mg). Methylated polysaccharide was extracted with $CHCl_3$ from this reaction mixture which showed no significant absorption band of OH in the 3500 cm^{-1} region in its IR spectrum.

11) E. Köiw and A. Grönwall, *Scan. J. Clin. Lab. Invest.*, **4**, 279 (1952).
12) L. Maraprade, *Bull. Soc. Chim. France*, **1**, 5, 833 (1934).
13) R.L. Whistler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
14) J.F. O'Dea and R.T. Gibbons, *Biochem. J.*, **55**, 580 (1953).

Reduction and Acetylation of O-Methyl Monosaccharide obtained from Methylated PF-I——The methylated PF-I (50 mg) was heated with 90% HCOOH in a boiling water bath for 4 hr. HCOOH was distilled off and the residue further hydrolysed with 1N H_2SO_4 for 4 hr in a boiling water bath.[15] The reaction mixture was neutralized with $BaCO_3$, and after passing through in a small column of Amberlite IR-120 (H^+), the clear filtrare was concentrated to a syrup. Then the mixture of O-methyl sugars was reduced in water (20 ml) with sodium borohydride (100 mg) for 12 hr. After treatment with Amberlite IR-120 and concentration, boric acid was removed by codistillation with methanol and the product treated with acetic anhydride–pyridine, 1: 1, (10 ml) at 100° for 15 min. The acetylation mixture was either injected into the column or was first diluted with water, concentrated to dryness and dissolved in acetone.

Gas-Liquid Partition Chromatography of Methyl O-Methyl Glycosides and O-Acetyl-O-Methyl Alditols obtained from Methylated PF-I——Methylated PF-I was converted into methyl glycosides by heating with 0.7N MeOH–HCl (5 ml) in a sealed tube for 5 hr in a boiling water bath. The solution was evaporated under atmospheric pressure to a syrup and HCl was removed by leaving it in a vacuum desicator over $CaCl_2$ and KOH pellets. Gas–liquid chromatography of the methyl O-methyl glycosides was effected in a Shimadzu GC-5A unit, equipped with a flame ionization detector, using a 200×0.3 cm glass column packed with 15% polybutane-1,4-diol succinate on celite 545 (60—80 mesh); column temperature, 175°; N_2 flow rate, 50 ml/min.

Gas–liquid chromatography of O-acetyl-O-methyl alditols derived from the methylated PF-I was carried out at the condition of gas flow rate of 50 ml N_2/min on a glass column (200×0.3 cm) containing 5% (w/w) of ECNSS on Chromosorb W (aw-dmcs, 60—80 mesh), at 180°.

Paper Chromatography and Paper Electrophoresis of Hydrolysate of Methylated PF-I——The methylated PF-I was treated by formic acid-dilute sulfuric acid method.[15] Paper chromatography of the hydrolysate of methylated PF-I was examined using AcOEt–AcOH–H_2O (9: 2: 2), and 3 spots detected corresponded to tetra-O-methyl, tri-O-methyl, and di-O-methyl monosaccharides. Di-O-methyl and tri-O-methyl monosaccharides were identified by paper electrophoresis using 1% $Na_2B_4O_7$ solution as 3,6-di-O-methyl-D-mannose (MG: 0.38) and 3,4,6-tri-O-methyl-D-mannose (MG: 0.25), 2,3,6-tri-O-methyl-D-galactose (MG: 0.0). Reference di-O-methyl mannoses had MG 0.09 (2,6-), 0.14 (2,3-), 0.43 (4,6-), 0.49 (3,4-), 0.39 (3,6-) and tri-O-methyl mannoses 0.24 (3,4,6-), 0.0 (2,3,6-), tri-O-methyl galactose 0.0 (2,3,6-), 0.28 (3,4,6-).

Component Sugars of Minor Polysaccharide (0.05M $NaHCO_3$ Fraction)——A part of the fraction was hydrolysed with 1N H_2SO_4 for 6 hr at 100°, and the resultant sugars were detected by paper chromatography as described above. Mannose and galactose, were detected.

15) H.O. Bouveng, H. Kiessling, B. Lindberg and J.E. McKay, *Acta. Chem. Scand.*, **16**, 615 (1962); H.O. Bouveng and B. Lindberg, "Methods in Carbohydrate Chemistry," Vol. 5, ed. by R.L. Whistler, Academic Press, Inc., New York, 1965, p. 297.