Summary

Capsular polysaccharide of the *Cryptococcus neoformans* was found to be a xyloglucuronomannan, which consisted of D-glucuronic acid, D-xylose, and D-mannose (in 1:1:3 ratio), and its molecular weight was about 6600 by the Akiya-Barger's method and from results of end-group assay. D-Xylose and a part of D-glucuronic acid in this polysaccharide were liberated more easily than D-mannose.

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130 Toshio Miyazaki : Studies on Fungal Polysaccharides. III.*1 Chemical Structure of the Capsular Polysaccharide from *Cryptococcus neoformans*.

(*Tokyo College of Pharmacy**2)

In the previous paper of this series,*1 it was shown that the polysaccharide of *Cryptococcus neoformans* consisted of D-xylose, D-glucuronic acid, and D-mannose in a ratio of 1:1:3, and hydrolysis of this polysaccharide liberated D-xylose and D-glucuronic acid more easily than D-mannose. In this paper, the results of further investigations on the chemical structure of this polysaccharide, based on periodate oxidation and methylation, will be described.

On periodate oxidation of potassium salt of the capsular polysaccharide, the consumption of periodate per 166 g. of the polysaccharide was as follows : 0.99 moles (after 6 hours), 1.24 (24 hours), 1.31 (48 hours), 1.38 (120 hours). The value of formic acid liberated from 166 g. of polysaccharide was 0.47 moles (after 20 hours), 0.55 (144 hours), and that of formaldehyde was 0.02 mole (after 216 hours) respectively.

The periodate-oxidized polysaccharide was reduced with sodium borohydride followed by acid hydrolysis. Paper chromatographic analysis of the hydrolysate revealed the presence of D-mannose as the only reducing sugar in addition to erythritol and a small amount of glycerol, and xylose and glucuronic acid were not detected.

These results, pointed to the following conclusions : (1) D-Xylose and D-glucuronic acid are not present as branching point of the polysaccharide. (2) A part of D-mannose would be the branching point or have 1:3 linkage. (3) Other part of D-mannose would have 1:4 linkage. (4) The value of formaldehyde liberated from the polysaccharide in periodate oxidation was equivalent to one mole per 45 moles hexose units.

It is known[1] that D-glucuronide consumes periodate in neutral medium more rapidly than other aldoses. In the case of this polysaccharide, in spite of the presence of non-oxidized mannose the rapid consumption of periodate in the initial stage of oxidation suggests that glucuronic acid would be present as the end group of this polysaccharide.

The polysaccharide was methylated several times with dimethyl sulfate and sodium hydroxide, and further repeatedly with methyl iodide and silver oxide. During the course

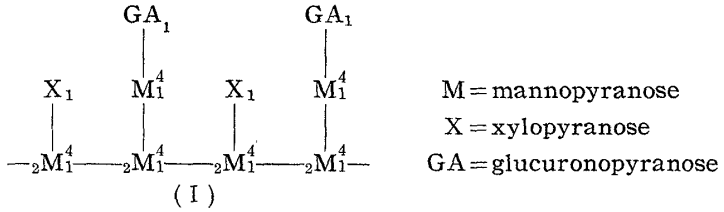
*1 Part II : This Bulletin, **9**, 826 (1961).
*2 Kashiwagi 4-chome, Shinjuku-ku, Tokyo (宮崎利夫).
1) R. A. Eddington, E. L. Hirst, E. E. Percival : J. Chem. Soc., **1955**, 2281.

of this experiment, acetylation with pyridine and acetic anhydride on this partially methylated polysaccharide and remethylation were carried out alternatively to effect complete methylation. The fully methylated polysaccharide (pale yellow powder, $-OCH_3$ 43.3%) was hydrolyzed with formic acid followed by *N* sulfuric acid. The neutralized hydrolysate was passed through ion-exchange resin (Amberlite IR-120) column and the effluent was concentrated to a syrup. The methylated monosaccharide in the hydrolysate was examined by paper chromatography and paper electrophoresis.

Paper chromatogram of this hydrolysate, using the solvent systems of (1) butanol-ethanol-water (4:1:5), (2) butanol-acetic acid-water (4:1:1), (4) butanol-pyridine-water (10:3:3), and (4) ethyl acetate-acetic acid-water (9:2:2) revealed the presence of 2,3,4-tri-O-methyl-D-xylose, 2,3,4-tri-O-methyl-D-glucuronic acid, 2,3,6-tri-O-methyl-D-mannose, and dimethyl-D-mannose. These results are shown in Table I. Both Rg*[3] value and Mg*[4] value of the dimethylmannose in the hydrolysate were found to differ distinctly from those of authentic 2,3-, 3,4-, and 4,6-dimethyl-D-mannose. Furthermore, the Mg value of dimethylmannose in the hydrolysate differed markedly from that of 2,6-dimethylmannose reported by Stacey, *et al.*[2] Although unequivocal identification of dimethylmannose was unsuccessful, because 2,4- and 3,6-dimethylmannose have not been synthesized yet, it was concluded from above experiments that the dimethylmannose in the hydrolysate was 2,4- or 3,6-dimethyl-D-mannose.

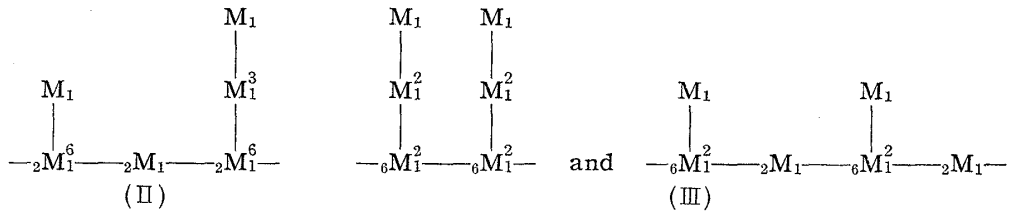
Quantitative analysis of each of the methylated monosaccharides in the polysaccharide by Hirst's method[3] showed that it consisted of 2,3,4-tri-O-methyl-D-xylose, 2,3,4-tri-O-methyl-D-glucuronic acid, 2,3,6-tri-O-methyl-D-mannose, and 3,6(or 2,4)-dimethyl-D-mannose in a molecular ratio of 1:1:1:2.

From the foregoing series of experiments and data on the partial hydrolysis of the polysaccharide,*[1] and from the standpoint of prevailing uniformity of the mode of linkage in natural polysaccharides, the capsular polysaccharide might contain a most probable partial structure represented by (I).



(I)

It is interesting that this structure (I) of the capsular polysaccharide from *C. neoformans*, a kind of yeast-like fungi, resembles those of yeast mannan[4] (II) and mannan[5] (III) from *Saccharomyces rouxii*.



(II) (III)

*[3] $Rg = \dfrac{\text{Rf of a sample}}{\text{Rf of 2,3,4,6-tetramethyl-D-glucose}}$

*[4] $Mg = \dfrac{\text{true distance of migration of a sample}}{\text{true distance of migration of D-glucose}}$

2) S. A. Barker, A. B. Foster, I. R. Siddiqui, M. Stacey: J. Chem. Soc., **1958**, 2358.
3) E. L. Hirst, L. Hough, J. K. N. Jones: *Ibid.*, **1949**, 928.
4) W. N. Haworth, R. L. Heath, S. Peat: *Ibid.*, **1941**, 833.
5) P. A. J. Gorin, A. S. Perlin: Canad. J. Chem., **35**, 262 (1957).

Experimental

Periodate Oxidation of the Polysaccharide——The polysaccharide (K salt, 14.6 mg.) was dissolved in a small volume of 3% NaCl solution and its total volume was made up to 25 cc. with 3 cc. of 0.25*M* $NaIO_4$ and 3% NaCl solution. This mixture was allowed to stand in the dark at room temperature and the determinations of the consumption of periodate, HCOOH, and HCHO produced was carried out with this solution by the procedure of Akiya and Tomoda.[6] The number of moles of $NaIO_4$ consumed per 166 g. of the polysaccharide was : 0.71 (after 1 hr.), 0.91 (3 hr.), 0.99 (6 hr.), 1.24 (24 hr.), 1.31 (48 hr.), 1.33 (96 hr.), 1.38 (120 hr.).

The corresponding number of moles of HCOOH produced was : 0.47 (after 20 hr.), 0.55 (144 hr.), and HCHO produced was 0.022 mole (after 216 hr.).

Estimation of the Periodate-Oxidation Products——The polysaccharide (100 mg.) was oxidized with $NaIO_4$ as described above. After 144 hr., ethylene glycol (5 cc.) was added to destroy the excess periodate and the reaction mixture was dialyzed against running water. $NaBH_4$ (100 mg.) was added to the non-dialyzable solution with vigorous stirring and the stirring was continued for 7 hr. After standing overnight, excess $NaBH_4$ was destroyed by acidification with AcOH, the reaction mixture was concentrated to dryness, and heated with 2*N* H_2SO_4 (10 cc.) for 4 hr. on a boiling water bath. The hydrolysate was neutralized with $BaCO_3$, centrifuged, the supernatant was passed through a column of ion-exchange resin, Amberlite IR-120 (H), and Amberlite IR-4B (OH). The effluent was concentrated to a small volume *in vacuo* and examined by paper chromatography. Rf : 0.20 (BuOH-AcOH-H_2O=4:1:5 (5), Toyo Roshi No. 51, 3% *p*-anisidine (BuOH) spray, D-mannose 0.20), 0.17 (AcOEt-AcOH-H_2O=3:1:3 (6); D-mannose 0.17), 0.45 (PhOH-H_2O=3:1, D-mannose 0.45). This syrup was extracted with MeOH, MeOH was evaporated from the extract, and the residue was examined by paper chromatography and paper electrophoresis, the result of which is shown in Table I.

Table I. Rf and Mg values of the Reduced Oxidation-Products

Substance \ Solvent system	(5)	(6)	Mg[*5]
D-Mannose	0.20	0.17	0.69
Erythritol	0.29	0.29	0.75
Glycerol	0.39	0.40	0.56
Sample	0.20	0.17	0.69
	0.29	0.29	0.75
	0.39 (trace)	0.40 (trace)	0.56 (trace)

Solvent system : (5) BuOH-AcOH-H_2O (4-1-5)
(6) AcOEt-AcOH-H_2O (3-1-3)
Detection by HIO_4-benzidine spray[7]

Methylation of the Polysaccharide——Purified polysaccharide (K salt, 1.0 g.) was dissolved in H_2O (20 cc.), 30% NaOH (45 cc.) and Me_2SO_4 (15 cc.) were added drowise with vigorous stirring and water-cooling, at such a rate that the mixture remained slightly alkaline. After 4.5 hr., the reaction mixture was allowed to warm to room temperature, and 30% NaOH (134 cc.) and Me_2SO_4 (45 cc.) were added dropwise over 4 hr. with stirring continued for 7 hr. The same quantities of the reagents as above were added over 2 hr., and the stirring was continued further for 42 hr. The reaction mixture was heated on a boiling water bath for 30 min. to destroy the excess of Me_2SO_4. The ice-cooled reaction mixture was acidified by the cautious addition of 10% H_2SO_4, and the partially methylated polysaccharide precipitated. The precipitate was collected on a sintered glass filter and extracted repeatedly with $CHCl_3$ and Me_2CO. The product thus obtained was methylated again under the above conditions. The methylated product, isolated through acidification of the reaction mixture, was further methylated by refluxing with MeI (10 cc.) containing Me_2CO (5 cc.) and Ag_2O (2 g.). Ag_2O was added in small portions over 3 hr. and the refluxing was continued further for 10 hr. The same treatment (without Me_2CO) was repeated eight times.

The final product was extracted with $CHCl_3$ from the reaction mixture (0.2 g., $-OCH_3$ 41.5%) and heated for 10 hr. with pyridine (4 cc.) and Ac_2O (2 cc.) at 75°. After standing overnight, the solvent was distilled off *in vacuo*, the residue was dissolved in Me_2CO (10 cc.), 30% NaOH (6.4 cc.) and Me_2SO_4 (2.4 cc.) were added dropwise over 1 hr. at 45°, and the stirring was continued for 3 hr. The reaction mixture was heated on a water bath at 60° for 30 min,. diluted with H_2O, acidified with dil. H_2SO_4,

*5 1% borax, 500 v./30 cm., 4 hr., Toyo Roshi No. 51.
6) S. Akiya, M. Tomoda : Yakugaku Zasshi, **76**, 575 (1956).
7) J. A. Cifonelli, F. Smith : Anal. Chem., **26**, 1132 (1954).

and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried over anhyd. Na_2SO_4, and the solvent was distilled off. The $CHCl_3$ residue was methylated under the same conditions and was further refluxed with MeI (5 cc.) and Ag_2O (2 g.) for 20 hr., twice. To the $CHCl_3$ solution of the final product, half its volume of hexane was added, and after removal of a small quantity of the precipitate by centrifugation, excess of hexane was added to the supernatant. The precipitate was centrifuged, dried *in vacuo* to a pale yellow powder. *Anal.* Calcd. for $(C_{43}H_{76}O_{26})_x$: $-OCH_3$, 43.5. Found : $-OCH_3$, 43.3.

Examination of the Methylated Monosaccharide——The methylated polysaccharide (120 mg.) was hydrolysed by heating with 90% HCOOH (12 cc.) for 10 hr. at 100° and, after removal of the HCOOH by heating with *N* H_2SO_4 (3 cc.), further for 4 hr. The hydrolysate was neutralized with $BaCO_3$, $BaSO_4$ was filtered off, and the filtrate was passed through a column of Amberlite IR-120 (H). The eluate, on concentration, gave the methylated monosaccharides as a syrup. The results of paper chromatographic examination of methylated monosaccharide in the syrup is shown in Table II and its Mg values on paper electrophoresis is in Table III.

Table II. Rg values of Methylated Monosaccharides

Monosaccharide \ Solvent	(1)	(2)	(3)	(4)
4,6-Di-O-methyl-D-mannose	0.62	0.69	0.69	0.51
2,3-Di-O-methyl-D-mannose	0.63	0.70	0.69	0.54
3,4-Di-O-methyl-D-mannose	0.64	0.72	0.71	0.55
2,3,6-Tri-O-methyl-D-mannose	0.83	0.89	0.88	0.85
3,4,6-Tri-O-methyl-D-mannose	0.84	0.90	0.88	0.86
2,3,4-Tri-O-methyl-D-mannose	0.85	0.91	0.92	0.87
2,3,4-Tri-O-methyl-D-xylose	0.94	0.96	0.98	1.01
2,3,4-Tri-O-methyl-D-glucuronic acid	0.32	0.86	0.24	0.89
2,3,4,6-Tetra-O-methyl-D-glucose	1.00	1.00	1.00	1.00
Methylated monosaccharide from the hydrolysate	0.32	0.73	0.24	0.57
	0.66	0.86	0.72	0.85
	0.83	0.89	0.89	0.89
	0.94	0.96	0.98	1.01

Solvent system : (1) BuOH-EtOH-H_2O (4 : 1 : 5)
(2) BuOH-AcOH-H_2O (4 : 1 : 1)
(3) BuOH-pyridine-H_2O (10 : 3 : 3)
(4) AcOEt-AcOH-H_2O (9 : 2 : 2)
Detection by 3% *p*-anisidine-hydrochloride (BuOH) spray

Table III. Mg values of Methylated Monosaccharides

	Mg		Mg
2,6-Di-O-methyl-D-mannose	0.09[2)]	2,3,4-Tri-O-methyl-D-xylose	0.00
2,3-Di-O-methyl-D-mannose	0.14	2,3,4-Tri-O-methyl-D-glucuronic acid	0.80
4,6-Di-O-methyl-D-mannose	0.43	2,3,4,6-Tetra-O-methyl-D-glucose	0.00
3,4-Di-O-methyl-D-mannose	0.49	Methylated monosaccharide	0.00
2,3,6-Tri-O-methyl-D-mannose	0.00	from the hydrolysate	0.39
3,4,6-Tri-O-methyl-D-mannose	0.05		0.80

1% Borax, 500 v./30 cm., 4 hr., Toyo Roshi No. 51.
Detection by 3% *p*-anisidine-hydrochloride (BuOH) spray

Quantitative Estimation of the Methylated Monosaccharides——Each of the spots separated on chromatogram of the methylated monosaccharide developed by solvent system (1) was quantitatively extracted with boiling MeOH and filtered through a glass filter. Each filtrate was concentrated to dryness, the residue was dissolved in H_2O (5 cc.), and estimated by Hirst's method. These results are shown in Table IV.

Table IV. Molar Ratio of the Methylated Monosaccharides

	Found (mg.)	Molar ratio
3,6-Di-O-methyl-D-mannose	3.96	2.16
2,3,6-Tri-O-methyl-D-mannose	2.11	1.15
2,3,4-Tri-O-methyl-D-xylose	1.83	1.00
2,3,4-Tri-O-methyl-D-glucuronic acid	1.89	1.03

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Summary

The chemical structure of the capsular polysaccharide from *Cryptococcus neoformans* was further investigated by periodate oxidation and methylation. Periodate oxidation of the polysaccharide produced formic acid and formaldehyde, and the xylose and glucuronic acid were attacked, but a part of mannose was resistant. Hydrolysis of the methylated polysaccharide gave 2,3,4-tri-O-methyl-D-xylose, 2,3,6-tri-O-methyl-D-monnose, 2,3,4-tri-O-methyl-D-glucuronic acid, and 3,6-di-O-methyl-D-mannose in 1:1:1:2 ratio. From these results, the chemical structure of this polysaccharide was proposed.

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