Studies on the Cell Wall of *Piricularia oryzae*

Part III. The Chemical Structure of the β -D-Glucan[†]

By Tasuku NAKAJIMA, Kinjiro TAMARI, Kazuo MATSUDA,
Hirosato TANAKA* and Nagahiro OGASAWARA*

Department of Agricultural Chemistry, Tohoku University, Sendai

**Department of Agricultural Chemistry, Niigata University, Niigata*

Received May 21, 1971

The cell wall β -D-glucan of *Piricularia oryzae* has been studied by methylation, periodate oxidation and fragmentation analysis.

Hydrolysis of the methylated D-glucan afforded 2,3,4,6-tetra- (1 mole), 2,4,6-tri- (9 moles), 2,3,4-tri- (2 moles) and 2,4-di-O-methyl-D-glucose (1 mole). The degraded glucan obtained after Smith degradation was also examined by methylation analysis. Methylation of the degraded glucan, followed by subsequent hydrolysis, gave 2,3,4,6-tetra- (1 mole) and 2,4,6-tri-O-methyl-D-glucose (10 moles).

Controlled acetolysis of the D-glucan afforded a homologous series of laminaridextrins, whereas enzymatic degradation with β -1,3 glucanase from *Basidiomycete* sp. QM 806 gave glucose, gentiobiose and gentiotriose.

Additional evidence for the structure of the D-glucan has been provided by periodate oxidation studies. The glucan was oxidized by periodate, consuming 0.36 mole of periodate and producing 0.18 mole of formic acid per anhydroglucose unit.

These results suggest that the cell wall β -D-glucan has a β -1,3 linked backbone with the side chains attached through β -1,6 linkages and a part of the backbone is constituted of β -1,6 linked residues.

Previous study^{1,2)} in this series has revealed that the cell wall of *Piricularia oryzae* was constituted of at least three different polysaccharides; heteropolysaccharide containing D-mannose, β -D-1,3 glucan, and chitin-like substance. Of the cell wall components, the β -D-1,3 glucan occupies more than 60%, while the contents of heteroglycan and chitin-like substance are 10% and 13%, respectively.

β -D-1,3 Glucan is well known to be major components of the cell walls of yeasts and fungi. The structure of the cell wall glucan of yeasts has been studied by several investigators.^{3~8)}

Recently, Misaki *et al.*⁹⁾ have reported on

the detailed structure of the cell wall glucan of *Saccharomyces cerevisiae*. Using the refined technique of methylation and periodate oxidation, they demonstrated that the above glucan had a highly branched structure of β -1,6-linked main chains with branches at C-3. However, little information is available on the detailed structure of the β -D-glucan of fungal cell wall, since the cell wall of fungi contains a considerable amount of chitin which interferes the separation of the glucan. Now we have succeeded to obtain pure β -D-glucan from the cell wall of *Piricularia oryzae* by exhaustive extraction with alkali followed by removal of chitin by chitinase digestion.

The present paper deals with the structural study of the alkali-insoluble cell wall glucan of *Piricularia oryzae*.

[†] This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, 1971.

EXPERIMENTAL

General methods. All evaporation were carried out under reduced pressure at 35~40°C. The melting points were uncorrected. Optical rotations were measured with a Nippon Bunko Model DIP-SL polarometer.

Paper chromatography was performed on Toyo No. 50 and No. 527 filter paper by descending or multiple ascending methods using following solvent systems (v/v): a) *n*-butanol, pyridine, water (6:4:3),¹⁰⁾ b) ethyl acetate, pyridine, water (10:4:3),⁹⁾ c) butanone-water azeotrop.¹¹⁾

Reducing sugars were detected on the chromatogram by spraying the paper with aniline hydrogen phthalate.¹²⁾

Gas liquid chromatography was carried out on a Yanagimoto Model G-8 gas chromatography apparatus fitted with a flame-ionization detector, using a glass column (300×0.3 cm) which was packed with 10% Poly (DEG) succinate on Diasolid. Analysis were made at 200°C, at a nitrogen flow rate of 12.5 ml/min.

Ultracentrifugation of a methylated glucan was conducted by a Hitachi UCA-1 analytical ultracentrifuge. Sedimentation was performed at 51,200 rpm for 1 hr in chloroform.

Complete acid hydrolysis of the oligosaccharides and their corresponding sugar alcohols was performed by heating the compounds (1~2 mg) in a sealed tube with 1 N sulfuric acid (0.5 ml) at 100°C. Partial acid hydrolysis was achieved by refluxing the compounds (1~2 mg) with 0.1 N hydrochloric acid (0.5 ml) on a boiling water bath for 1~2 hr. Reduction of the oligosaccharides into the corresponding sugar alcohols was achieved by treating the aqueous solution of the sugars (5 mg in 1 ml of water) with an equal amount of sodium borohydride at 30°C overnight. Amberlite IR-120 (H) resin was added to the reaction mixture until gas evolution ceased. The solution was diluted with water and stirred with an additional Amberlite IR-120 to remove sodium ion. The deionized solution was evaporated to dryness, and the residue was dissolved in anhydrous methanol and evaporated to dryness, the procedure being repeated three times.

Culture of Piricularia oryzae. Preparation of the mycelium was described in the previous paper.¹⁾

Isolation of the cell wall glucan. The freeze-dried and powdered mycelium (84 g) was dispersed in water (2 liters) and the mixture was heated at 120°C for 1 hr in an autoclave. After centrifugation at 6000×g, the insoluble residue was collected. The same proce-

dure was repeated once more. Then, the water insoluble residue was dispersed in 1 N sodium hydroxide and the mixture was shaken for 48 hr at room temperature. After centrifugation at 6000×g, the insoluble residue was collected. This alkali extraction was carried out two times more. The alkali insoluble residue, which was recovered by centrifugation, was neutralized with acetic acid and dialyzed overnight against tap water. The residue was collected by centrifugation, washed with hot water and freeze-dried. The cell wall material thus obtained (17 g) was then suspended in 500 ml of 0.01 M phosphate buffer (pH 6.5) and incubated with chitinase (8800 units) from *Streptomyces* sp. CH-9¹⁾ at 50°C for five days. The chitinase digestion was repeated once more. The residue of the digest was collected and washed repeatedly with water. The residue was again dispersed in water and heated at 120°C in an autoclave for 1 hr. After cooling, the insoluble D-glucan was collected, washed with water and freeze-dried. (Yield 9 g)

Properties of the cell wall glucan. Nitrogen was determined by a micro Kjeldahl method.¹³⁾

Complete acid hydrolysis of the glucan was performed in the following manner. The glucan (71 mg) was stirred with 25 N sulfuric acid (0.5 ml) at 2°C for 4 hr during which time the glucan was completely dissolved. Water (12 ml) was then added to make the concentration of the acid 1 N and the whole solution was heated at 100°C for 6 hr. The hydrolyzate was neutralized with barium carbonate, filtered and the filtrate was concentrated to a syrup. The paper chromatographic analysis (solvent A, B) revealed glucose only. The glucose content of the glucan as determined by the phenol-sulfuric acid method¹⁴⁾ was 97% of the theoretical value.

Periodate oxidation of the cell wall glucan and preparation of the degraded glucan. The cell wall glucan (385 mg) was dissolved in dimethyl sulfoxide (90 ml) by stirring at 60°C for 48 hr under nitrogen. Water (100 ml) was then added and the swollen glucan was collected by centrifugation. The collected glucan was dialyzed against deionized water for three days to remove trace of the dimethyl sulfoxide. The residual glucan was stirred with 0.05 M sodium metaperiodate (84.5 ml) at 5°C in the dark. At suitable intervals, aliquots of the reaction mixture were taken for the analysis. The periodate consumption was determined by spectrophotometric method¹⁵⁾ and formic acid was determined by potentiometric titration with 0.01 N

sodium hydroxide. The polyaldehyde was suspended in water (50 ml) and reduced with sodium borohydride (100 mg) according to the procedure of Smith *et al.*³²⁾ The excess of sodium borohydride was decomposed by careful acidification with hydrochloric acid. The concentration of the acid was adjusted to 0.1 N and the mixture was kept at room temperature for 48 hr. The modified polysaccharide was recovered by centrifuging and washed with water, and then freeze-dried. Degraded glucan (80 mg) was recovered.

Methylation of the cell wall glucan and the degraded glucan. The initial methylation was carried out by the method of Haworth.³⁰⁾ To a suspension of the glucan (2 g) in acetone, 30% aqueous sodium hydroxide and dimethyl sulfate were simultaneously added dropwise under vigorous stirring within 4 hr, during which time the whole mixture was kept at 50°C. The excess dimethyl sulfate was decomposed and the residual acetone was removed by raising the temperature to 85°C. The excess alkali was neutralized and the reaction mixture was dialyzed. The dialyzed material was concentrated to a small volume and the methylation was repeated twice in the same manner. (Yield 1.2 g).

Partially methylated glucan was further methylated four times by the method of Hakomori.³¹⁾ The methyl sulfinyl carb anion was prepared according to the procedure of Sandford *et al.*¹⁶⁾

After the sixth methylation, the product was extracted with chloroform. The chloroform extract was washed with water, dried over sodium sulfate and the solvent was evaporated. (Yield 700 mg, OCH_3 : 45.4%, $[\alpha]_D + 47^\circ$ ($c=1$ in chloroform))

The methylation of the degraded glucan (60 mg) was carried out four times by the method of Hakomori.³¹⁾ (Yield 35 mg)

Methanolysis and hydrolysis of the methylated glucan. For gas liquid chromatography, the methylated glucan (10 mg) was heated in a sealed tube with 0.85 N methanolic hydrogen chloride (1 ml) at 100°C for 24 hr. The methanolizate was neutralized with silver carbonate, filtered and evaporated. The residue was redissolved with a small amount of methanol and submitted to gas liquid chromatography.

For the identification of hydrolysis products of methylated glucan, it was hydrolyzed by the method of Garegg and Lindberg.¹⁷⁾ The methylated glucan (400 mg) was suspended in 25 N sulfuric acid (1 ml) and stirred at room temperature for 8 hr during which

time it was completely dissolved. Water was then added to bring the acid concentration to 2 N and the mixture was heated at 100°C for 8 hr. The hydrolyzate was neutralized with barium carbonate, filtered, deionized with Amberlite IR 120 (H) and concentrated. The hydrolyzate (173 mg) was separated on thick papers (Toyo No. 527) with solvent C. (Recovery 101%).

The identification of the components was made by direct comparison of the crystalline products.

A: 2,3,4,6-Tetra-O-methyl-D-glucose (13 mg) had mp and mixed mp 89°C, and $[\alpha]_D + 81^\circ$ ($c=0.5$ in water) (lit. mp 95~96°C and $[\alpha]_D + 83.8^\circ$ in water).¹⁸⁾

B: 2,4,6-Tri-O-methyl-D-glucose (130 mg) had mp and mixed mp 123°C, and $[\alpha]_D + 110^\circ \rightarrow 76^\circ$ ($c=1$ in water) (lit. mp 123°C and $[\alpha]_D + 111^\circ \rightarrow 70^\circ$ in water).¹⁹⁾

C: 2,3,4-Tri-O-methyl-D-glucose was not clearly identified but compared with an authentic sample by paper chromatography with solvent C and by gas liquid chromatography.

D: 2,4-Di-O-methyl-D-glucose (11 mg) had $[\alpha]_D + 36^\circ \rightarrow 75^\circ$ ($c=0.6$ in water) (lit. $[\alpha]_D + 76.5^\circ$ in water).²⁰⁾

Fragmentation of the cell wall glucan by acetolysis. The cell wall glucan (20 g) was acetolyzed following the method of Matsuda *et al.*,²¹⁾ described in the previous paper.²⁾

The acetolizate (16 g) was deacetylated with 0.05 N sodium methoxide and neutralized with Amberlite IR 120 (H), filtered and concentrated. (Yield 7 g).

The deacetylated acetolizate (5 g) was dissolved in water (50 ml) and poured on a chacoal-celite (No. 545) (1:1 w/w) column (7×10 cm), and fractionated by stepwise elution with water and ethanol. The effluent was collected in 500 ml fractions and each fraction was examined by paper chromatography with solvent B. Chromatographically identical fractions were combined and concentrated to dryness, and then dissolved in hot methanol. The solution was filtered and reevaporated to dryness.

The identification of the components was made by direct comparison of the crystalline products.

A: 3-O- β -D-Glucopyranosyl-D-glucose (laminaribiose) (600 mg). The fractions 20~39 eluted with 2.5~5% ethanol, were chromatographically identical with laminaribiose. The crystalline sugar had mp and mixed mp 203°C, and $[\alpha]_D + 22^\circ$ ($c=2$ in water) (lit. mp 196°C and $[\alpha]_D + 20^\circ$ in water).²²⁾

Acetylation with acetic anhydride and sodium acetate afforded the β -octa-acetate which had mp and mixed mp 161°C, and $[\alpha]_D - 28^\circ$ ($c=0.25$ in chloroform) (lit. mp 161°C and $[\alpha]_D - 28^\circ$ in chloroform).²³⁾

B: O- β -D-Glucopyranosyl-(1 $\rightarrow$ 3)-O- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-D-glucose (laminaritriose) (126 mg). The fractions 51~67 eluted with 15~20% ethanol were chromatographically identical with laminaritriose. The syrup had $[\alpha]_D + 6^\circ$ ($c=2$ in water) (lit. $[\alpha]_D + 2.4^\circ$ in water),²⁴⁾ and β -hendeca-acetate had mp 116°C and $[\alpha]_D - 37^\circ$ ($c=0.2$ in chloroform) (lit. mp 120°C and $[\alpha]_D - 40^\circ$ in chloroform).²⁴⁾

Fragmentation by enzymatic degradation. The enzyme was prepared from a culture of *Basidiomycete* sp. QM 806 (kindly provided by Dr. S. Kikumoto)²⁵⁾ following the method of Reese and Mandels.³³⁾ This enzyme preparation was 30 units/mg on determination by the method of Reese *et al.*³³⁾ and had a negligible amount of other β -glucanase activity except β -1,3 glucanase activity.

The cell wall glucan (3.5 g) was suspended in citrate buffer (pH 4.8, 0.05 M, 200 ml) and incubated with the crude β -1,3 glucanase (900 units) at 55°C for 24 hr. The digest was centrifuged and the supernatant was deionized by passing through Amberlite IR 120 (H) and Amberlite IRA 400 (OH) resins, and concentrated to a syrup. (Yield 1.4 g). The syrup was separated by chromatography on thick papers (Toyo No. 527) with solvent A. The identification of the components was made by direct comparison of the crystalline products.

A: 6-O- β -D-Glucopyranosyl-D-glucose (gentiobiose) (108 mg) had $[\alpha]_D + 14^\circ$ ($c=2$ in water) (lit. $[\alpha]_D + 10.5^\circ$ in water),²⁶⁾ and β -octa-acetate had mp and mixed mp 192°C, and $[\alpha]_D - 4^\circ$ ($c=0.5$ in chloroform) (lit. mp 195°C and $[\alpha]_D - 5^\circ$ in chloroform).²⁷⁾

B: O- β -D-Glucopyranosyl-(1 $\rightarrow$ 6)-O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-glucose (gentiotriose) (164 mg) had mp 148°C and $[\alpha]_D - 8^\circ$ ($c=1$ in water) (lit. $[\alpha]_D - 10.4^\circ$ in water),²⁸⁾ and β -hendeca-acetate had mp 213°C, $[\alpha]_D - 9^\circ$ ($c=0.2$ in chloroform) (lit. mp 214°C and $[\alpha]_D - 9.4^\circ$ in chloroform).²⁸⁾ Partial acid hydrolysis of this sugar gave glucose and gentiobiose as reducing sugar. Reduction of this compound with sodium borohydride followed by partial acid hydrolysis afforded glucitol and gentiobitol as sugar alcohol, and glucose and gentiobiose as reducing sugar.

Determination of number average molecular weight. We used a modified method of Jones and Ballou.²⁹⁾ The cell wall glucan (about 10 mg) was swollen with dimethyl sulfoxide (DMSO) and the DMSO was removed by dialyzing before examination. Dextran T-20 (Mn 15000) (about 10 mg) and gentiotetraose

(about 5 mg) were dissolved in 0.5 ml of water, respectively. To each sample solution was added 0.5 ml of a sodium borohydride-borotritide mixture (25 μ Ci/ml). The mixtures were kept at 40°C for 3 hr with shaking. Into small test tubes containing the mixture, were put Amberlite IR 120 (H) resins which was packed in dialyzing tubes. After 1-hr shaking, the dialyzing tubes were taken off and washed with water. The reaction mixture was concentrated to dryness and washed twice with methanol, and evaporated to dryness. The reduced samples were purified by gel filtration (gentiotetraitol) on a Sephadex G-25 column and by dialysis (reduced cell wall glucan and Dextran T-20). The purified samples were hydrolyzed in 2 N sulfuric acid or by another method for hydrolyzing cell wall glucan described before. The hydrolyzates were assayed for carbohydrate and radioactivity.

RESULTS AND DISCUSSION

A water insoluble β -D-glucan was isolated from the cell wall of *Piricularia oryzae*. Protein, glycogen and heteroglycan were removed by exhaustive extraction with superheated water and 1 N sodium hydroxide. Chitin was removed by treatment with chitinase from *Streptomyces* sp.¹⁾ After freeze-drying and grinding, the glucan was obtained as a white powder (N: 0.06%) which was insoluble in water and alkali, but slightly soluble in dimethyl sulfoxide.

The molecular weight of the glucan was determined by the procedure of Jones and Ballou,²⁹⁾ which involves sodium borotritide reduction of the glucan and analysis of specific activities of a standard oligosaccharide and the reduced glucan. The results of analysis are shown in Table I. From the specific activities of gentiotetraitol and reduced glucan, a number average molecular weight of about 38,500 was calculated for the *P. oryzae* cell wall β -D-glucan. The dextran T-20 was used for a control polysaccharide.

Methylation of the cell wall glucan first with dimethyl sulfate and alkali,³⁰⁾ and then with methyl iodide in the presense of methylsulfinyl carbanion³¹⁾ yielded the fully methylated glucan (OCH₃: 45.4%) in 35% yield. The methylated

TABLE I. INCORPORATION OF TRITIUM FROM SODIUM BOROTRITIDE INTO REDUCING ENDS OF GENTIOTETRAOSE AND CELL WALL GLUCAN OF *Piricularia oryzae*.

Compound reduced	Tritium incorporated cpm	Specific activity cpm/100 μ g	Molecular weight
Gentiotetraose	53,981	5,742	666
Dextran T-20 ^a (M.W. 15,000)	2,780	241	15,811
Cell wall glucan	496	99	38,491

a) Pharmacia Catalogue, Uppsala, Sweden. (Dextran T-20, $\bar{M}_n=15,000$, $\bar{M}_w=22,300$)

glucan showed $[\alpha]_D +47^\circ$ (in chloroform) and a single peak on ultracentrifugation analysis.

Hydrolysis of the methylated glucan with sulfuric acid afforded a mixture of methylated sugars which were shown by paper chromatography to consist of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri- and 2,4-di-O-methyl-D-glucose. The quantitative estimation of these methyl derivatives was carried out by separating the hydrolyzate on paperchromatogram and also by gas liquid chromatography of the methanolyzate. The molar ratio of these O-methyl sugars was 1:9:2:1.

Methylation analysis was also applied to the degraded glucan obtained by the Smith degradation³²⁾ involving periodate oxidation, reduction and mild acid hydrolysis. In this case, the glucan was devoid of 2,3,4-tri and 2,4-di-O-methyl derivatives, and the molar ratio of 2,3,4,6-tetra and 2,4,6-tri-O-methyl-D-glucose was 1 : 10.

According to these results of methylation analysis as shown in Table II, the structure of degraded glucan will be shown as (I),

and then the original glucan will be given as (II).

TABLE II. HYDROLYSIS PRODUCTS OF METHYLATED,
ORIGINAL AND DEGRADED CELL WALL GLUCAM
OF *Piricularia oryzae*.

O-Methyl-D-glucose		Molecular ratio			
		Original glucan		Degraded glucan	
		G.L.C.	P.P.C.	G.L.C.	P.P.C.
2,3,4,6-Tetra-	G ¹⁻	1	1	1	1
2,4,6-Tri-	⁻³ G ¹⁻	9	—	10	10
2,3,4-Tri-	⁻⁶ G ¹⁻	2	11	0	0
2,4-Di-	⁻⁶ ₃ G ¹⁻	—	1	—	0

G.L.C., gas liquid chromatography

P.P.C., paper partition chromatography

When the cell wall glucan was treated with periodate at 5°C, 0.36 mole of periodate was consumed and 0.18 mole of formic acid was produced per anhydroglucose unit as shown in Fig. 1. This indicates that two molar proportion of periodate were consumed with the simultaneous liberation of one molar proportion of formic acid, for an average of

FIG. 1. Periodate Oxidation of the Cell Wall Glucan of *Piricularia oryzae*.

○—○: Periodate consumed,
▲—▲: Formic acid produced.

six residues of D-glucose. This result is obviously incompatible with the result of methylation analysis. However, this contradiction can be accounted for by an assumption that a part of the 1,6-linkage is located in the backbone of the glucan. This view was also supported by the results of fragmentation analysis.

The controlled acetolysis of the cell wall glucan afforded a homologous series of laminaridextrin, such as glucose, laminaribiose and laminaritriose. On the other hand, enzymatic digestion of the glucan with an exo-type β -D-1,3 glucanase from *Basidiomycete* sp. QM 806^{33,34)} afforded a mixture of gentiobiose and gentiotriose.

The simplest possible structure proposed for the cell wall glucan of *Piricularia oryzae*, based on the results of methylation, periodate oxidation and fragmentation analysis is shown in Fig. 2.

FIG. 2. The Possible Structures Proposed for the Cell Wall Glucan of *Piricularia oryzae*.

G→G denotes an β -glucosidic linkage.

The proposed structures above mentioned are in harmony with our previous results of the selective enzymatic degradation of *Piricularia oryzae* cell wall by lytic enzymes from *Bacillus circulans*.¹¹⁾ The cell wall was digested by β -1,3-glucanase with a substantial decrease in turbidity but slight liberation of reducing sugar, while the action of β -1,6 glucanase resulted in liberation of a considerable amount of reducing sugar although the concomitant decrease in turbidity was only slight. These results suggest that the cell wall of *Piricularia oryzae* is mainly constituted of the β -D-1,3-glucan

as networks and β -1,6-glucan as fillings, or the latter constitutes short side chains of the backbone of the β -1,3-glucan.

Our present results strongly support the latter idea, although the presence of a glucan of different types cannot be completely neglected.

Acknowledgements. The authors wish to thank Dr. S. Kikumoto, Taito Co., Ltd., for a gift of *Basidiomycete* sp. QM 806 strain and enzymes. Our thanks are also due to Dr. Y. Kamio for his kind help in gas liquid chromatography. This investigation was supported in part by a grant-in-aid for scientific research from the Ministry of Education.

REFERENCES

- 1) H. Tanaka, N. Ogasawara, T. Nakajima and K. Tamari, *J. Gen. Appl. Microbiol.*, **16**, 39 (1970).
- 2) T. Nakajima, K. Tamari, K. Matsuda, H. Tanaka and N. Ogasawara, *Agr. Biol. Chem.*, **34**, 553 (1970).
- 3) L. Zechmeister and G. Toth, *Biochem. Z.*, **270**, 309 (1934).
- 4) W. Z. Hassid, M. A. Joslyn and McCready, *J. Am. Chem. Soc.*, **63**, 295 (1941).
- 5) D. J. Bell and D. H. Northcote, *J. Chem. Soc.*, **1950**, 1944.
- 6) S. Peat, W. J. Whelan and T. E. Edwards, *ibid.*, **1958**, 3862.
- 7) D. J. Manners and J. C. Patterson, *Biochem. J.*, **98**, 19c (1966).
- 8) C. T. Bishop, F. Blank and P. E. Gardner, *Can. J. Chem.*, **38**, 869 (1960).
- 9) A. Misaki, J. Johnson, Jr. S. Kirkwood, J. V. Scaletti and F. Smith, *Carbohydr. Res.*, **6**, 150 (1968).
- 10) A. Jeans, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).
- 11) L. Boggs, L. S. Cuendet, I. Ehrenthal, R. Koch and F. Smith, *Nature*, **166**, 520 (1950).
- 12) S. M. Partridge, *ibid.*, **164**, 443 (1949).
- 13) A. C. Chibnall, M. W. Rees and E. F. Williams, *Biochem. J.*, **37**, 354 (1943).
- 14) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 15) G. O. Aspinall and R. J. Ferrier, *Chem. Ind. (London)*, **1957** 1216.
- 16) P. A. Sandford and H. E. Conrad, *Biochemistry*, **5**, 1508 (1966).

- 17) P. J. Garegg and B. Lindberg, *Acta Chem. Scand.*, **14**, 871 (1960).
 - 18) J. C. Arvine and J. W. H. Oldham, *J. Chem. Soc.*, **119**, 1744 (1921).
 - 19) C. C. Baker, E. L. Hirst and J. K. N. Jones, *ibid.*, **1938**, 1695.
 - 20) J. W. VanCleve and W. C. Schaefer, *J. Am. Chem. Soc.*, **77**, 5341 (1955).
 - 21) K. Matsuda, H. Watanabe and K. Aso, *Nippon Nogeikagaku Kaishi*, **35**, 1227 (1961).
 - 22) S. A. Warsi and W. J. Whelan, *Chem. Ind. (London)*, **1957**, 1573.
 - 23) P. Bachli and E. G. Percival, *J. Chem. Soc.*, **1952**, 1243.
 - 24) S. Peat, W. J. Whelan and H. G. Lawley, *ibid.*, **1958**, 724.
 - 25) S. Kikumoto, T. Miyajima, K. Kimura, S. Okubo and N. Komatsu, *Nippon Nogeikagaku Kaishi*, **45**, 162 (1971).
 - 26) A. Thompson and M. L. Wolfrom, *J. Am. Chem. Soc.*, **75**, 3605 (1953).
 - 27) M. L. Wolfrom, A. Thompson and A. M. Brownstein, *ibid.*, **80**, 2015 (1958).
 - 28) S. Haq and W. J. Whelan, *J. Chem. Soc.*, **1956**, 4543.
 - 29) G. H. Jones and C. E. Ballou, *J. Biol. Chem.*, **244**, 1052 (1969).
 - 30) W. N. Haworth, E. L. Hirst and H. A. Thomas, *J. Chem. Soc.*, **1931**, 821.
 - 31) S. Hakomori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
 - 32) I. J. Goldstein, G. W. Hay, B. A. Lewis and F. Smith, "Methods in Carbohydrate Chemistry," Vol. V, Academic Press Inc., New York and London, 1965, p. 361.
 - 33) E. T. Reese and M. Mandels, *Can. J. Microbiol.*, **5**, 173 (1959).
 - 34) T. E. Nelson, J. Johnson, Jr., E. Jantzen and S. Kirkwood, *J. Biol. Chem.*, **244**, 5972 (1969).
-