BIKEN JOURNAL Vol. 12, 231-244, 1969

A HIGHLY BRANCHED α-D-GLUCAN OF *VIBRIO PARAHAEMOLYTICUS* A55.
I. ITS ISOLATION AND STRUCTURE

TOSHIHIDE TAMURA and TSUNESABURO FUJINO

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka

HIDEKI MIYAJI and AKIRA MISAKI

Laboratory of Applied Biochemistry, Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka

SHOZO KOTANI

Department of Microbiology, Osaka University Dental School, Osaka
(Received September 19, 1969)

SUMMARY An unusual type of α-1,4-D-glucan (V-glucan) was found in the envelope fraction of *Vibrio parahaemolyticus* A55. V-glucan ($[\alpha]_D^{20}=+150$, in water) was obtained by digestion of the envelope fraction from this organism with L-11 enzyme to degrade the peptidoglycan and then precipitation of the digest with 50 % ethanol. Methylation, peroxidation and Smith degradation studies revealed that it was an α-1, 4-D-glucan with an extremly high degree of branching at the C-6 position. This glucan differs from so-called glycogen in that it is not iodophilic and is virtually insensitive to α- or β-amylase, but it is degraded by glycogen-debranching enzymes, such as *Pseudomonas* isoamylase and pullulanase, releasing maltotriose and maltotetraose as the major products. The possible structure of this glucan and its location in the cell are discussed on the basis of these findings.

INTRODUCTION

Glycogen-like polysaccharides have been isolated from various bacteria and fungi (Dawes et al., 1964; Gorin et al., 1968). In earlier investigations, the characterizations of these polysaccharides of microbial and animal origins involved iodine staining, examination of the specific optical rotation, opalescent appearance in aqueous solution and degradation by β-amylase. Recently, analyses by methylation and periodate oxidation have also been used to elucidate the structural features of these polysaccharides. All informations available suggest that the glycogen-like polysaccharides isolated from microbial cells have a structure very similar to that of animal glycogen.

The envelope fraction of *Vibrio parahaemolyticus* A55, was found to contain a polysaccharide composed solely of D-glucose residues. Leutgeb and Weidel (1963) described a glycogen-like polysaccharide, associated with the ' rigid-

layer' of *Escherichia coli* B, which was easily removed by digestion with α-amylase, leaving an intact 'bag-shaped' peptidoglycan. However, all attempts to remove the polysaccharide from the peptidoglycan fraction of the *Vibrio* by α-amylolysis were unsuccessful. Furthermore, the polysaccharide failed to give a reddish brown color, characteristic of glycogen, when the envelope fraction was treated with iodine, though it gave a positive reaction with PAS (Periodic Acid-Schiff) stain. Thus the question arose of whether this material was actually a glycogen-type polysaccharide and of whether it was a reserve material or a structural constituent of the envelope. The present paper reports the structural features of the α-glucan of *Vibrio parahaemolyticus* A55, tentatively named V-glucan, elucidated by methylation, periodateoxidation, Smith degradation and enzymatic studies.

MATERIALS AND METHODS

1. *Organism and cultivation*

A strain (A55, serotype O5 : K15) of *Vibrio parahaemolyticus* originally isolated from a case with food poisoning in Yokohama, Japan, by Takikawa (1958) was used throughout the study. The strain was generously supplied by Dr. G. Omori, Osaka City Institute of Hygiene, and was kept as a stab culture in our laboratory by serial monthly transfer in medium of the composition described below supplemented with 0.5% agar. On cultivation, the stab culture was spread on an EMB agar (Eiken Chemicals Co. Ltd., Tokyo) plate supplemented with 3% NaCl, adjusted to pH 7.8 with NaOH and organisms forming smooth colonies were selected for seed culture. Every lot of seed culture (grown under the same conditions as the bulk culture but for 16 hr) was checked by the slide agglutination test with anti-K15 rabbit serum (purchased from Toshiba Chemicals Co. Ltd., Tokyo). Eight ml of the seed culture were inoculated into a 500 ml-flask containing 200 ml of medium, composed of 1% each of casamino acid (Nissan, Nissui Seiyaku Co. Ltd., Tokyo), yeast extract (Oriental Yeast Co. Ltd., Tokyo), maltose (Nakarai Chemicals Co. Ltd., Kyoto) and 3% NaCl, adjusted to pH 7.8 with NaOH. Flasks were incubated with vigorous shaking at 37 C. Cells were harvested by centrifugation under cooling in the late exponential phase of growth (usually 4 or 5 hr after inoculation) and were put in a deep-freeze.

2. *Isolation of the envelope fraction*

As summarized in Fig. 1, the envelope fraction was obtained by treating the cells successively with sodium dodecyl sulfate (SDS), urea and trypsin, according to the method of Kolenbrander and Ensign (1968) with some modification. Details of the procedure have been published (Tamura et al., 1968; Tamura, 1969).

3. *Isolation of V-glucan from the envelope fraction*

The envelope fraction was digested with L-11 enzyme (Kotani et al., 1959; Kato et al., 1962) (Fig. 1). This enzyme preparation was a gift from Dr. Y. Hirachi and was shown to lyse *Staphylococcus aureus* cell walls due to its *N*-acetylmuramyl-L-alanine amidase and glycine bridge splitting enzyme activities (Kato et al., 1968).

A portion (550 mg) of the envelope fraction was incubated with 1,200 units of L-11 enzyme in 50 ml of 0.125 M phosphate buffer, pH 8.0, at 37 C for 48 hr. The digest was centrifuged at 5,000 *g* for 30 min and the precipitate, which has been shown to consist of poly-β-hydroxybutyrate granules (PHBA (Tamura et al., 1968), was washed with water by centrifugation. The supernatants were combined and concentrated to about 10 ml in a rotary evaporator. Then absolute alcohol was added dropwise with continuous stirring until the final concentration of ethanol was 50%. The white precipitate formed was collected by centrifugation, redissolved in 10 ml of water and reprecipitated with 50% ethanol. The supernatants obtained after treatment with 50% ethanol were combined, concentrated and lyophilized for further study.

4. *Analytical methods*

The total amount of NH_2-terminals was determined after their conversion to dinitrophenylated derivatives, as described by Ghuysen et al., (1966) with minor modifications. Total hexosamines were determined by the method of Roseman and Daffner (1956). Hexose and reducing sugars were estimated by the anthrone method (Aschwell, 1957) and by the method of Park and Johnson (1949), respectively. Unless otherwise stated, all these methods were carried out on a micro-scale, using a Hitachi Perkin-

Vibrio parahaemolyticus A55 (92 g dry weight)

Suspended in 2 liter of 1 % SDS solution containing 3 % NaCl. Incubated at 37C for 20 hr with shaking. 1 mg DNAase added to reduce viscosity. Centrifuged at 58,000 *g* for 60 min at room temperature. Sediment submitted to a second treatment with SDS.

Precipitate 1

Suspended in 1 liter of 6 M urea solution and kept at 37C for 16 hr. Centrifuged as above. Precipitate washed with deionized water by cetrifugation untill SDS completely removed.

Precipitate 2

Digestion with 10 mg crystalline trypsin in 100 ml of 0.02 M tris-HCl buffer at pH 7.0, for 4 hr at 37C. Digestion repeated. Centrifuged as above. Precipitate washed with deionized water by centrifugation.

Precipitate 3
(Envelope fraction, 5 g dry weight)

Envelope fraction (550 mg)

Digested with 1,200 units of L-11 enzyme in 50 ml of 0.0125 M phosphate buffer, pH 8.0, at 37C for 48 hr. Centrifuged at 5,000 *g* for 30 min. Precipitate washed with deionized water. Supernatant combined with washing water.

Precipitate (220 mg, PHBA granules)

Supernatant

Concentrated in a rotary evaporator to about 10 ml. Absolute ethanol added to a final concentration of 50 %. Precipitation formed dissolved in deionized water and reprecipitated with 50 % ethanol. Precipitate collected by low speed centrifugation.

Precipitate (170 mg, V-glucan)

Supernatant (120 mg, fragements of peptidoglycan with non-precipitated glucan)

FIGURE 1. *Procedure for isolation of the envelope fraction and V-glucan of Vibrio parahaemolyticus A55.*

Elmer Spectrophotometer (Model 139 UV-VIS, Hitachi Ltd., Tokyo) with a quartz micro-cell of 300 μl capacity.

5. *Gas-liquid phase chromatography*

Gas chromatograms were run in a Hitachi Chromatograph, Model KGL-2B, equipped with a flame-ionization detector, in a stainless steel column (100 × 0.4 cm inside diameter).

6. *Hydrolysis of V-glucan and identification of its constituent*

A sample of V-glucan was hydrolyzed with 2N H_2SO_4 for 4 hr at 100 C and then passed through an Amberite IR4B(OH^-) column. Te eluate with water was concentrated and examined by paper chromatography (on Toyo Roshi No. 51 paper, with *n*-propanol-ethylacetate-water, 7 : 1 : 2, v/v, as the solvent system descending technique for 16 hr at room temperature), by paper electrophoresis (on Whatman 3MM paper, in 10% NaB_4O_7, pH 9.0 at 600 v with a current of 10 ma, for 2.5 hr at room temperature) and by gas-liquid chromatography (with a column of 1.5% Silicone Gum SE-52 on Chromosorb W-AW-DMCS, under N_2 gas at a flow rate of 60 ml/min, by the method of Sweeley, 1962). Sugars on the paper chromatogram were detected by spraying the paper with alkaline silver nitrate or *p*-anisidine hydrochloride in water-saturated *n*-butanol. For paper electrophoretograms were sprayed with *p*-anisidine-trichloroacetate.

7. *Periodate oxidation*

A sample (20 mg) of V-glucan was dissolved in 15 ml of 0.01 M sodium meta-periodate. Oxidation was carried out in the dark at room temperature. At suitable intervals, aliquots were removed (1 ml for estimation of periodate consumption and 2 ml for measurement of formic acid production). The Periodate consumption was determined by the usual arsenite method (Fleury and Lange, 1933) and formic acid production was estimated by titration with 0.01 N sodium hydroxide. For reference, rabbit liver glycogen (Calbiochem Co. Ltd., Los Angeles U.S.A.) and α-methylglucoside were similarly oxidized.

8. *Methylation*

The Methylation of the polysaccharides was performed by Hakomori's method (1964). Twenty mg of V-glucan were dissolved in 2 ml dimethylsulfoxide (DMSO) at room temperature and 0.5 ml of methylsulfonylcarbanion freshly prepared by the method of Sandford and Conrad (1966), was added dropwisely.

The reaction mixture was stirred mechanically under the N_2 atmosphere for 4 hr at room temperature. Then 0.5 ml methyl iodide was gradually added to the polysaccharide alkoxide over period of a few minutes, under cooling in a ice bath maintaining the reaction temperature at below 25 C. Then, the reaction mixture was tightly capped, stirred overnight at room temperature and dialyzed against running tap water. The methylated V-glucan in the dialyzate was extracted with chloroform, dehydrated in a molecular sieve (Linde type 4A), concentrated on a rotary evaporator and dried *in vacuo*. On infrared analysis, the material obtained showed no absorbtion at 3,600 cm^{-1}, indicating that most of hydroxyl groups had been replaced by methoxyl groups.

9. *Methanolysis and hydrolysis of methylated V-glucan*

The methylated product (8 mg) was methanolyzed by treatment with 2 ml of 5% methanolic hydrogen chloride in a sealed tube at 100 C for 18 hr. The methanolyzate was neutralized with Ag_2CO_3, filtered and concentrated. The syrupy residue was dissolved in methanol and examined by gas-liquid chromatography (using a column of 15% butanediol succinate on Neosorb NC, Nishio Ind. Co. Ltd., Tokyo, at 190 C, with N_2 gas at a flow rate of 60 ml/min).

The mixture of methyl-glucosides was hydrolyzed with 1 N H_2SO_4 at 100 C under reflux for 24 hr. The hydrolyzate was neutralized with $BaCO_3$, extracted with methanol and evaporated to dryness. The methylated glucose thus obtained was examined by paper chromatography (on Toyo Roshi No. 51 paper, with butanone-water-azeotrope, as the solvent system using the descending technique for 6 hr at room temperature).

10. *Smith degradation*

Following the method of Hamilton and Smith (1956), 20 mg of V-glucan were subjected to periodate oxidation with 12 ml of 0.1 M sodium metaperiodate at room temperature in the dark. After 25 days oxidation, 0.16 ml ethyleneglycol was added to the reaction mixture to decompose excess periodate and then the mixture was dialyzed. The oxidized glucan in the dialyzate was converted to the glucan-polyalcohol by addition of 20 mg of sodium borohydride, the reduction being completed after 24 hr at room temperature. After decomposition of the excess reagent with acetic acid, the polyalcohol was refluxed with 1 N H_2SO_4 for 6 hr. The hydrolyzate was neutralized with $BaCO_3$, filtered and the filtrate was concentrated to a syrup. The products were examined by paper chromatography (on Toyo Roshi No. 51 paper, with *n*-butanol-pyridine-water, 6 : 4 : 3 (v/v)). A portion of the hydrolyzate was converted to the trimethylsilyl (TMS) derivative by the method of Sweeley (1962) or to the acetyl derivative by the method of Sawardeker et al. (1965). These derivatives were examined by gas chromatography under the following conditions; for the TMS-derivative, UCON-LB550X, at 105 C with N_2 at a flow rate of 40 ml/min; for the acetylated sample, 3% ECNSSM 100–200 mesh, at 200 C with N_2 at a gas flow vate of 10 ml/min.

11. *Iodine reaction*

Two mg of V-glucan or rabbit liver glycogen were dissolved in 10 ml of 0.01 N HCl solution (pH 2–3), containing 0.04% iodine and 0.4% potassium iodite. Samples of 0.2 mg of amylopectin (Nakarai Chemicals Col. Ltd., Kyoto), dextran (Meito Co. Ltd., Nagoya), potato starch (Nakarai Chemicals Co. Ltd., Kyoto) or β-limit dextrin of rabbit liver glycogen (prepared in our laboratory) were dissolved in 10 ml of 0.01 N HCl containing 0.004% iodine and 0.04% potassium iodite. The light absorptions of these solutions at 350–700 mμ were measured in a Hitachi Spectrophotometer, Model 124, against iodine-iodite solution.

12. *Enzymatic degradation*

Samples of each of 1 mg of V-glucan were incubated with 20 μg of crystalline α-amylase (from *Bacillus subtilis*, Sigma Chemicals Co. Ltd., St. Louis. U.S.A) in. 1 ml of 0.02 M phosphate buffer, pH 6.9, containing 0.006 M NaCl, or 20 μg of crystalline β-amylase (from sweet potato, Sigma) in 1 ml of 0.01 M acetate buffer, pH 5. *Pseudomonas* isoamylase and pullulanase were kindly supplied by Mr. Yokobayashi and used according to the indication of Yokobayashi et al., (1969). The isoamylase was purified from the culture supernatant of *Pseudomonas sp.*, strain SB15, by Harada Yokobayashi and Misaki (1968) and was demonstrated to hydrolyze all α-1,6 glucosidic interchain linkages in amylopectin and glycogen. However, the α-1,6 glucosidic linkage attaching a maltose residue to a chain of α-1,4 linked glucose residues, was not readily hydrolyzed by this enzyme (Yokobayashi, Misaki and Harada, 1969). Pullulanase, from *Aerobacter aerogenes* ACTT 9621, is known to

cleave α-1,6 linked branch points of amylopectin, but its action on glycogen is limited to the outer-most chains.

13. *Analytical ultracentrifugation*

Analyses of 0.5% agueous solutions of samples were made in a Hitachi Analytical Ultracentrifuge, Model UCA-1, running at 18,270 rev/min. Photographs were taken at 3 min intervals.

14. *Infrared spectra*

These were measured in a KBr disk with a Hitachi Infrared Spectrophotometer, Model Ep1-2.

RESULTS

1. *Isolation of V-glucan from the envelope fraction using L-11 enzyme.*

V-glucan could be ' dissociated ' from other constituents of the envelope fraction such as peptidoglycan and PHBA granules, by digestion with L-11 enzyme (Fig. 1). Analysis of NH_2-terminal amino acids liberated during the enzyme digestion showed that the envelope fraction was degraded with release of 0.7 mole of NH_2-terminal alanine per 1.0 mole of glutamic acid residues. No significant increase in NH_2-terminal groups of other amino acids, including mono-NH_2-α, α-diaminopimeric acid (DAP), was observed. The result suggests that the degradation of the envelope fraction resulted from solubilization of its peptidoglycan component by *N*-acetylmuramyl-L-alanine amidase in the L-11 enzyme preparation (Tamura, 1969).

The digest of the envelope fraction with L-11 enzyme was centrifuged, and PHBA granules, previously shown by electron microscopy to be enclosed in a bag-shaped membrane, were precipitated. The supernatant which showed bright pale opalescence with Tyndall scattering, was fractionated by precipitation with 50 % ethanol. As shown Table 1, 60 % of the polysaccharide, expressed as hexose in the envelope fraction, was recovered in the precipitate. The bulk of the amino acids (estimated in terms of NH_2-groups) and amino sugars remained in the supernatant with a considerable amount of the polysaccharide. Precipitation with a higher concentration of ethanol may give a higher yield of polysaccharide, but at the same time may result in possible contamination of the peptidoglycan component. Therefore, in the present study 50 % ethanol was used to precipitate the polysaccharide to give minimal contamination of the peptidoglycan. The precipitated polysaccharide was tentatively named '*Vibrio*-glucan' abbreviated to V-glucan. The polysaccharide in the supernatant was found to be essentially similar to V-glucan in its poor affinity with iodine and its weak susceptibilities to α-and β-amylases but it was not studied further.

2. *Chemical and physical properties of V-glucan*

Paper chromatography, paper electrophoresis and gas chromatography showed that glucose was the sole component of V-glucan. The

TABLE 1. *Chemical compositions of the supernatant and precipitated fraction obtained from the L-11 enzyme digest of the envelope fraction by 50 % ethanol fractionation.*

Factions with 50 % ethanol	Total NH_2-groups (as glutamate)	Hexosamine (as glucosamine)	Hexose (as glucose, by anthrone method)	Glucose (by glucostat)[a]	Total reducing value (as glucose)[a]
	mμmole/mg				
Supernatant	1,030	50	5,230	—	—
Precipitate (V-glucan)	3.4	6.4	5,400	5,600	5,400

a The sample was hydrolyzed with 2N H_2SO_4 at 100 C for 4 hr.

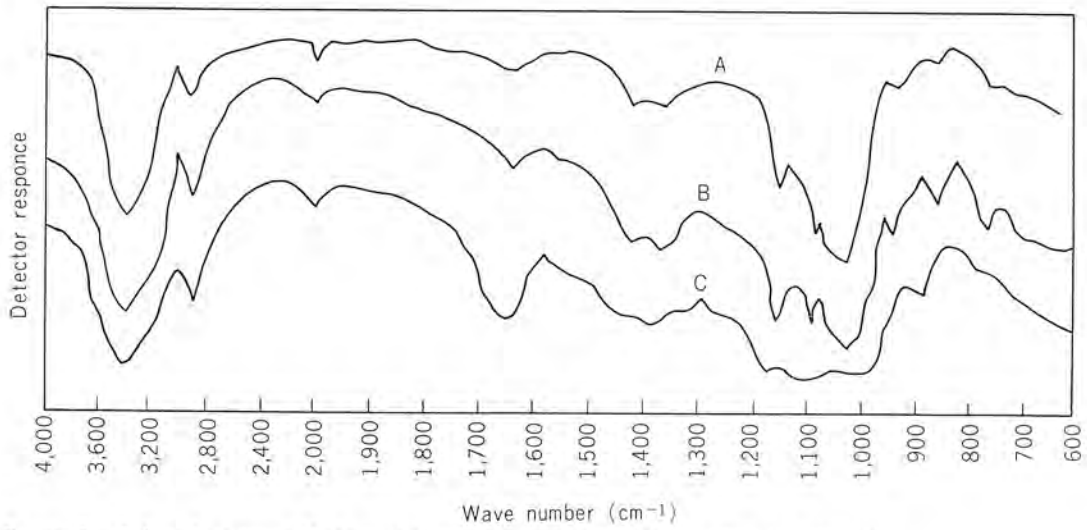

FIGURE 2. *Infrared spectra of V-glucan (A), rabbit liver glycogen (B) and yeast β-glucan (C).*

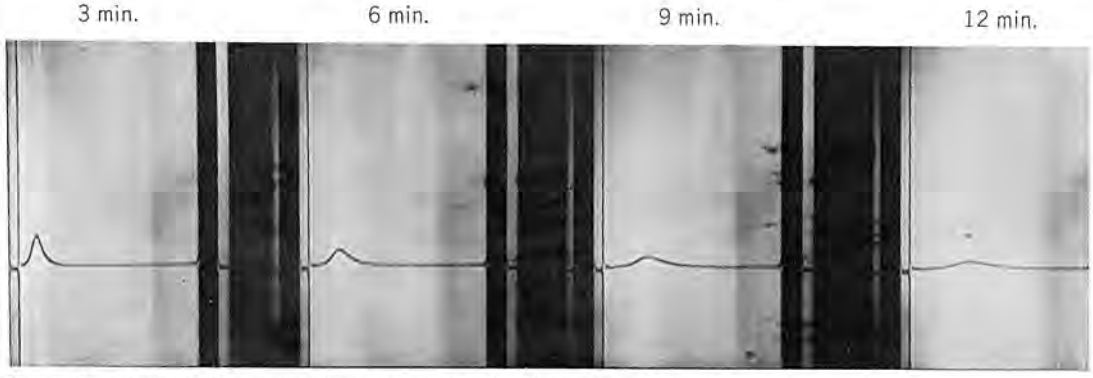

FIGURE 3. *Analytical ultracentrifugal pattern of a* 0.5% *aqueous solution of V-glucan. Sedimentation from left to right. The time after reaching* 18,270 *rev/min is indicated.*

glucose content of the acid hydrolyzate, estimated by the anthrone method, agreed with that obtained by the glucose oxidase method, confirming that the V-glucan was a pure glucan.

The infrared absorption spectrum of the V-glucan (Fig. 2) had absorption band at 840 cm^{-1} which were characteristic for α-linked polysaccharides and was identical with that of rabbit liver glycogen but apparently different from that of yeast β-glucan. The optical rotation of the V-glucan was $[\alpha]_D^{20}=+150$ in water, which is slightly lower than that

TABLE 2. *Light absorption characteristics of V-glucan, rabbit liver glycogen and related polysaccharides*

Test material	λ_{max}	E_{max}
V-glucan	450	0.006
Rabbit liver glycogen	470	0.12
Dextran	370	0.01
Amylopectin	560	0.16
β-limit dextrin[a]	535	0.20
Starch (potato)	600	0.30

a derived from rabbit liver glycogen

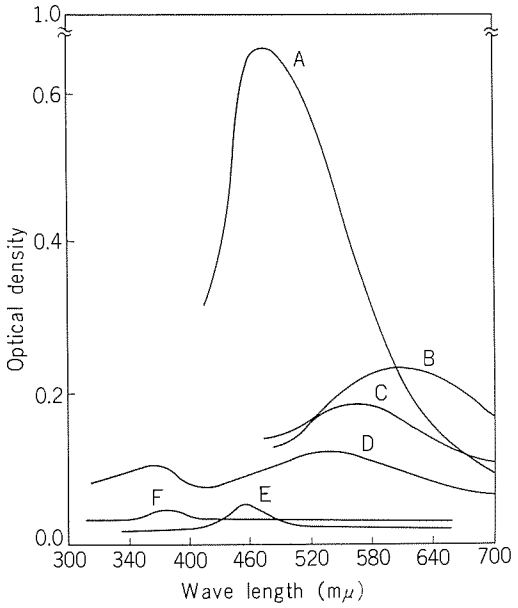

FIGURE 4. *Absorption spectra of polysaccharide-iodine complexes.*

A: rabbit liver glycogen. B: starch. C: amylopectin. D: β-limit dextrin. E: V-glucan. F: dextran. The concentration of V-glucan (E) and rabbit liver glycogen. (A) in iodine-iodite solution are ten times those of the other polysaccharide (see text).

of rabbit liver glycogen ($[\alpha]_D^{20}=+188$). The V-glucan gave a single peak at $S_{20W}=200S$ on sedimentation analysis but it appeared to be polydispersed because of the rapid spreading of its boundaries (Fig. 3).

3. *Light absorption spectra of V-glucan after treatment with iodine*

A wide absorption band at 460 mμ—480 mμ was obtained ($E_{max}=0.12$) with authentic rabbit liver glycogen. Unlike glycogen, the V-glucan gave an extemely weak extinction ($\lambda_{max}=450$, $E_{max}=0.006$). None of reference samples, potato starch, dextran, amylopectin or β-limit-dextrin gave spectra identical with V-glucan under the conditions used.

4. *Methylation*

Gas-liquid chromatography of the methanolysis products of the fully methylated V-glucan

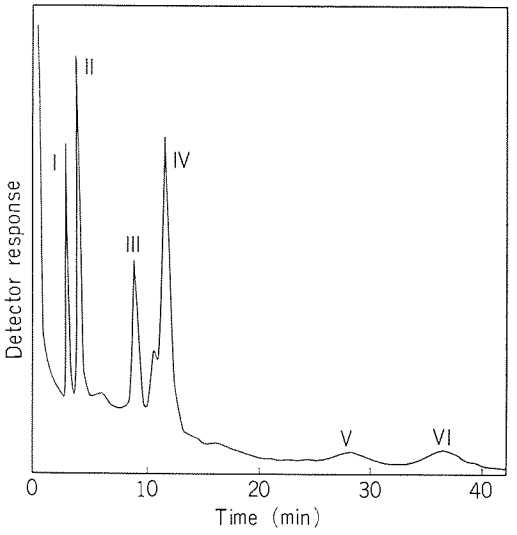

FIGURE 5. *Gas-liquid chromatogram of the methanolysis products of methylated V-glucan.*

I, II: methyl 2,3,4,6-*tetra-O-methyl-*D-*glucoside. III, IV: methyl* 2,3,6-*tri-O-methyl-*D-*glucoside. V, VI: methyl* 2,3-*di-O-methyl-*D-*glucoside. I, III and V are β anomers of the glycosides. II, IV and VI are α anomers.*

revealed (Fig. 5) the presence of two major components: methyl-2,3,4,6,-tetra-O-methyl-D-glucosides (peaks I and II, T_G, 0.71 and 1.00, respectively), and methyl-2,3,6-tri-O-methyl-D-glucoside (Peaks III and IV, T_G, 2,29 and 3.03, respectively).

(The retention times of methylglucosides (T_G) are expressed relative to that of α-methyl-3,3,4,6-tetra-O-methyl-D-glucosides.)

Methyl-2,3-di-O-methyl-D-glucoside, also detected on the gas chromatogram (Peak V and VI, T_G, 7.37 and 9.32, respectively) could not be measured quantitatively because the component giving these peaks were insufficiently volatile under the conditions used. The molar ratio is of tetra-O-methyl- to tri-O-methylglucoside was approximately 1 to 2.5. The paper chromatogram of the hydrolyzate of the products on methanolysis showed one major sport corresponding to 2,3,6-tri-O-methyl-D-glucose (R_F 0.55) together with two spots corresponding to 2,3,4,6,-tetra-O-methyl-D-glucose

(R_F 0.75) and 2,3-di-O-methyl-D-glucose (R_F 0.28), respectively. The presence of 2,3,6-tri-O-methyl glucose as the major component clearly indicates that the main type of linkage of V-glucan is, indeed, the 1,4 type. 2,3,4,6,-Tetra-O-methyl-D-glucose shoude be derived from the non-reducing terminals of V-glucan. 2,3-Di-O-methyl glucose must be from the branch points joined through C-1, C-4 and C-6 position. Theoretically, there should be equimolar amounts of di-O-methyl glucose and 2,3,4,6,-tetra-O-methyl glucoside, if the polysaccharide has a branched structure, and this was found to be case with V-glucan where 2,3-di-O-methyl- and 2,3,4,-6 tetra-O-methyl glucose gave spots of almost equal density on the paper chromatogram. The approximate molar ratio of tetra-: tri-: di-O-methyl-D-glucose of 1: 2.5: 1 indicates that V-glucan has a highly branched structure with repeating units consisting two or three 1,4 linked sugar residues, one nonreducing residue and one 1,6 linked branched residue.

5. *Smith degradation*

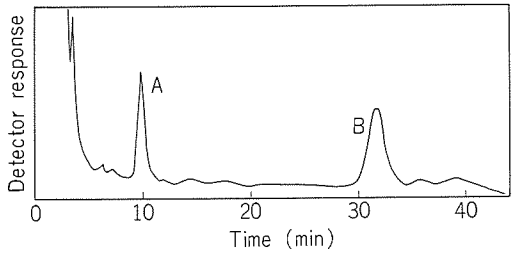

FIGURE 6. *Gas-liquid chromatogram of Smith degradation products of V-glucan.*
A: glycerol. B: erythritol. Degradation products were converted to their acetyl derivatives for analysis.

Paper chromatography of the products of Smith degradation gave two spots, one corresponding to erythritol and the other to glycerol. Quantitative analysis by gas-liquid chromatography of the TMS derivatives and acetyl derivatives (Fig. 6) showed that the molar ratio of erythritol to glycerol was approximately 3.5: 1.

TABLE 3. *Comparison of periodate consumption and formic acid production by periodate oxidation of V-glucan and rabbit liver glycogen*

Test material	Time of periodate oxidation		
	48 hr	72 hr	6 days
	moles/anhydroglucose		
V-glucan			
periodate consumption	1.14	1.25	1.36
formic acid production	0.22	0.24	0.25
Rabbit liver glycogen			
periodate consumption	0.97	0.94	1.09
formic acid production	0.07	0.09	0.09
α-methyl glucoside			
periodate consumption	2.07	2.00	2.13
formic acid production	0.84	0.83	0.99

6. *Periodate oxidation*

The consumption of periodate and production of formic acid on oxidation of V-glucan, rabbit liver glycogen and α-methylglucoside with 0.01 M sodium periodate at room temperature are shown in Table 3. Extrapolation of the values on the graph to zero time (Manners et al., 1961) gave net values for periodate consumption and formic acid production of 1.26 and 0.24 moles per mole of anhydroglucose for V-glucan. Assuming that V-glucan is an α-1,4 linked glucan with α-1,6 branch points and that the formic acid arises only from the non-reducing terminals, the above results indicate that the average length of the repeating units of V-glucan is about four to five. Under the same conditions, rabbit liver glycogen reduced 1.086 mole of periodate per anhydroglucose with the liberation of 0.085 mole of formic acid indicating that it had repeating units of 13 glucose residues. One mole of α-methylglucoside was found to reduce approximately 2 moles of periodate and produce one mole of formic acid under the same conditions, indicating that the oxidation was complete in 6 day at room temperature.

TABLE 4. *Action of amylolytic enzymes on V-glucan and rabbit liver glycogen*

Treatment	Liberation of reducing groups			
	V-glucan		Rabbit liver glycogen	
	mμmole/mg	(%)	mμmole/mg	(%)
Pullulanase	1,700	(32)[a]	60	(1)[a]
Isoamylase	2,000	(37)[a]	380	(6)[a]
β-amylase	0		1,200	(20)[a] (40)[b]
α-amylase	0		2,000	(33)[a] (66)[b]
Pullulanase and β-amylase[c]	2,600	(48)[a] (96)[b]	2,800	(47)[a] (94)[b]
Isoamylase and β-amylase[c]	3,800	(70)[a] (140)[b]	3,200	(53)[a] (106)[b]
V-glucan[d]	5,400	(100)[a]	6,000	(100)[a]

a Percentage of total reducing value.
b Expressed as maltose.
c Incubated simultaneously.
d Hydrolyzed with 2N H_2SO_4 at 100C for 4 hr.

7. *Action of amylolytic enzymes on V-glucan*

The actions of various hydrolytic enzymes on V-glucan and rabbit liver glycogen were assayed by measuring the increase in reducing power (Table 4). The amount of potential or total reducing termini was determined on samples hydrolyzed with 2 N H_2SO_4 at 100 C for 2 hr. The actions of α- and β-amylase on V-glucan did not lead to appreciable increases of reducing power, though gel filtration on Sephadex G-50 showed that treatment of the envelope fraction with either α- or β-amylase released about 2 % of the total carbohydrate of the envelope fraction, which was presumably maltose. About 20 % of the reducing termini of rabbit liver glycogen were released by β-amylolysis and 33 % by α-amylolysis. V-glucan was found to be susceptible to *Pseudomonas* isoamylase which released 37 % of its reducing termini and during the treatment the opalescent aqueous solution of V-glucan became clear. Pullulanase also attacked V-glucan, but unlike with isoamylase, during treatment it opalescence did not disappear.

Paper chromatograms of the digests with both isoamylase and pullulanase indicated the presence of mainly maltotriose and maltotetraose, but no maltose was detected. A small amount of maltopentaose was found in the isoamylase digest. Pullulanase seemed have less effect on V-glucan, since appreciable amounts of higher saccharides remained at the origin on the paper chromatogram. Both isoamylase and pullulanase caused complete degradation of V-glucan by simultaneous action with β-amylase. Debranching enzymes had much less effect on rabbit liver glycogen than on V-glucan, as judged by increase in reducing power. Paper chromatographic analysis also revealed that enzymic digests of glycogen contained oligosaccharides with longer chain lengths than those obtained with V-glucan.

DISCUSSION

Methylation and periodate oxidation of *Vibrio* polysaccharide (V-glucan) indicated that it is an extremely highly branched α-1,4 glucan, with repeating units with an average of 4 to 5 D-glucose residues and one sugar residue doubly substituted at the C–4 and C–6 positions to form a side chain. These results were supported by those of the Smith degradation. The molar ratio of erythritol to glycerol of 3.5: 1, obtained by the Smith degradation of V-glucan agreed well with the results of the methylation

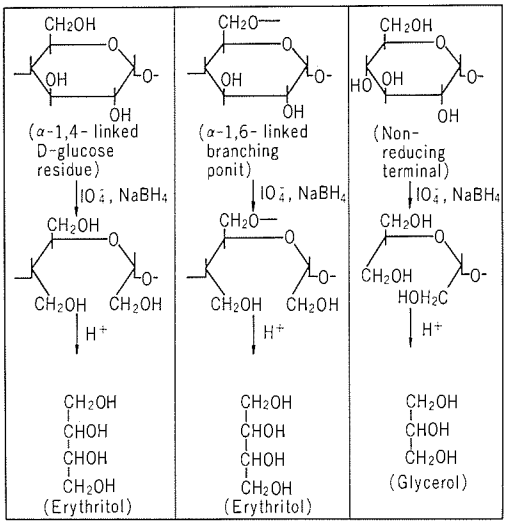

FIGURE 7. *Scheme of Smith degradation of starch-glycogen type polysaccharide.*

study. As shown Fig. 7, glycerol arises from the nonreducing terminal, and erythritol from the α-1,4 linked residues as well as the branching residues. The specific optical rotation ($[\alpha]_D^{20}=+150$) of V-glucan, and its infrared spectrum, are similar to those of glycogen or amylopectin but different from that of yeast β-glucan, indicating that the D-glucosidic bonds must be of the α-type. It should be pointed out, however, that this polysaccharide gave no reddish brown color with iodine, characteristic to a glycogen-type glucan, and that the degree of branching is two or three fold that of other known animal and microbial glycogens.

These findings prompted further characterization of V-glucan using various amylolytic enzymes. Studies on the actions of these enzymes on V-glucan and of their degradation products gave information on the molecular structure, particularly the mode of attachment of the side chains. It is known that the action of pullulanase, which hydrolyzed the outermost α-1,6 glucosidic interchain linkages in glycogen, required at least two α-1,4 linked glucose residues joined to the main chain (Abdullah et al., 1966), whilst *Pseudomonas* isoamylase, which cleaves all the branching points in glycogen requries a minimum of three glucose residues attached to the main chain. Therefore, V-glucan must have a structure such that it is split into larger proportions of maltotriose and maltotetraose with relatively small amounts of maltopentaose by the glycogen-debranching enzymes. Moreover all the α-1–4 linkages of V-glucan must be arranged in such a way that is hardly cleaved by α- or β-amylase. From these facts three possible structural models can be conceived for V-glucan. As shown in Fig. 8. model A represents a comb type structure, where almost all the α-1,4 linked glucose residues in the main chain carry side chains containing 3 or 4 glucose residues. However, this model does not explain the insusceptibility of V-glucan to β-amylase. In model B (laminated type) or model C (tree type), none of the chains would be attacked by β-amylase though it would be cleaved by debranching enzymes liberating maltotriose and maltotetraose. Furthur diagnostic data are necessary before a choice can be made between models A, B and C for the structure of V-glucan. If V-glucan is a componet of the cell envelope, the comb-type structure would be the most probable, as in the case of β-glucan of yeast cell walls in which numerous β-1,3 linked side chains are attached to the β-1,6 linked main chain (Misaki et al., 1968). However, if V-glucan is a reserve polysaccharide located inside of the envelope, its basic structure may resemble that of a glycogen type polysaccharide, supporting the multiply branched structure of model C. If V-glucan has the structure of model C it may be regarded as a starch-glycogen type polysaccahride, although it has a much shorter chain length and many more branches than ordinary glycogens. However, V-glucan differs with normal glycogens in the following respects: first, unlike known glycogens, it is almost insensitive to α- or β-amylase, but is fairly sensitive to debranching enzymes, indicating that it has a very high degree of branching. Second, it has a very weak affinity for iodine. This may be explained by the fact that the inter-

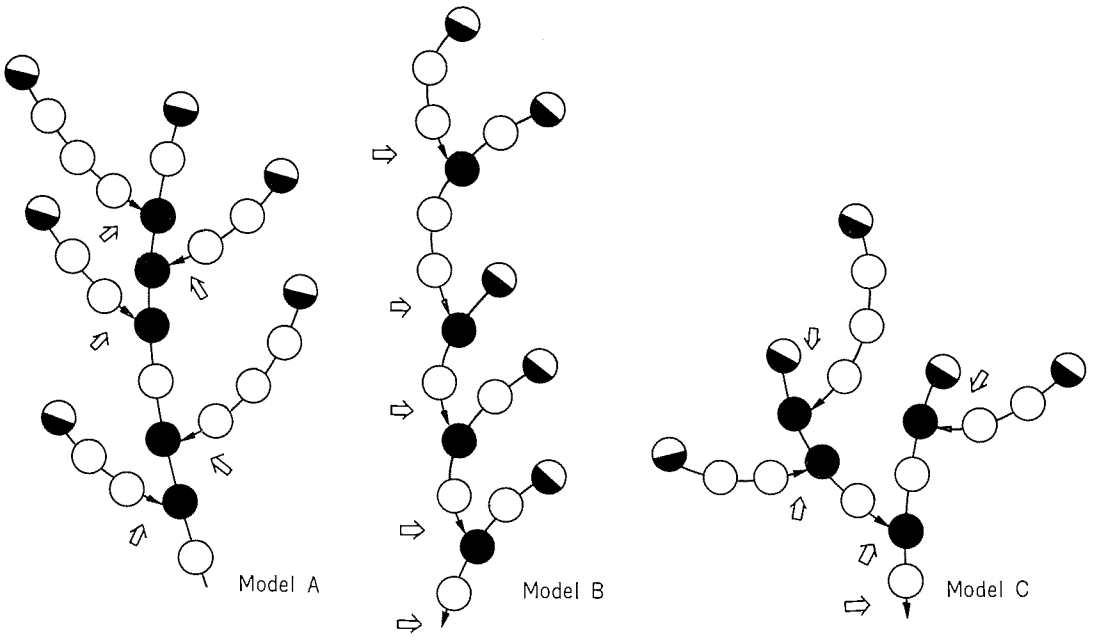

FIGURE 8. *Possible structure of V-glucan.* ○●◑ *glucose residue* ○– *α-1,4-linkage* ○→ *α-1,6-linkage* ● *Branch point* ◑ *Non-reducing terminal* ⇩ *Site of action of debranching enzyme* *Model A represents 'comb type'. Model B, 'laminated type'. Model C, 'tree type'.*

action of iodine and the glycogen molecule involves formation of an inclusion complex with the exterior chains of the latter containing an α-helix of six glucose residues (Manners, 1957) and the chains of V-glucan may be too short to form such a complex with iodine. Archibald et al., (1961) found a glycogen in human liver which had a weak affinity for iodine (E_{max}=0.05), but the affinity of V-glucan was even weaker (E_{max}=0.006). A glycogen-like α-glucan with an average chain length of 6–7 was isolated from the cell walls of BCG (Misaki et al., 1966). However, it was iodophilic, unlike V-glucan. No description has ever appeared of a similar type of α-glucan so far as the authors are aware.

It should be noted that V-glucan was isolated by a very mild method involving treatment with detergent and a peptidoglycan-degrading enzyme. This mild method would not cause any significant degradation or aggregation of molecules of the polysaccharide. Ultracentrifugal analysis showed that the molecular size ($S_{20,w}$=200 S) of V-glucan was compatible with those of glycogen molecules of large particle size (Golin et al., 1968).

Leutgeb and Weidel (1963) concluded that the glycogen-like polysaccharide in the envelope fraction of *E. coli* B neither contributed to the integrity of the cell wall nor had any structural association with the cell wall peptidoglycan, since it was easily removed by digestion with α-amylase without affecting the bag-shape of peptidoglycan. Holme et al., (1956) reported that a glycogen-like polysaccharide was accumulated and utilized in *E. coli* B under certain cultural conditions, suggesting that it was not a structural element of the cell. Microcyst walls of *Polysphondylium pallidum* prepared by sonic treatment contain approximately equal amounts of two glucans: cellulose and an alkali-soluble glucan assumed

to be glycogen (Toma et al., 1967). Cellulose and a glycogen-like polysaccharide were found in spore walls of *Dictyostelium discoideum* prepared by alkali extraction (Ward et al., 1965). However, the extent to which cytoplasmic glycogen may have contributed to their fractions is uncertain. Although there is no definite evidence that V-glucan is not associated with the cell envelope, it is very probable that such large particles as those of V-glucan, like PHBA granules, even if they are not associated with the membrane would remain inside of bag-shaped and not escape during the processes of isolation of the envelope fraction. It should be pointed out, in this connection, that in the present study the envelope fraction was prepared without any mechanical disruption of the cells. Further studies are necessary to see whether V-glucan serves as a metabolic substrate. If so, *Vibrio parahaemolyticus* would offer an intersting example in addition to those of *Rhodospirillum rubrum* (Stanier et al., 1959) and some species of *Pseudomonas* of simultaneous storage of two kinds of polymer as reserve materials. Systematic studies on the relationship between these two kinds of reserve materials seems to be scanty.

The question arises of whether V-glucan is a new type of polysaccharide or merely a variation or deformation of an ordinary type of glycogen caused by unbalance action or deficit of some enzyme system involved in its systhesis. If V-glucan has a tree type structure, it may be colsely related to the ' core ' of glycogen molecule from which glucose chains are extended. It would be possible that V-glucan is an immature form of glycogen or, in other words, a kind of ' primer ' of glycogen into which glucose residues are incorporated forming ' mature ' glycogen. Another possibility is that V-glucan may be a partially degraded glycogen formed by autolysis during the isolation procedure (Tanaka et al., 1969, reported that *V. parahaemolyticus* produced an extracellular amylase). However, this is unlikely because SDS treatment during the isolation procedure would inactivate autolytic enzymes, as Weidel et al. (1963) pointed out.

This report is the first case for *Pseudomonas* isoamylase to be used for structural study of a glycogen type polysaccharide. Recently, the contribution of various hydrolytic enzymes to the structural analysis of glycogen and starch has become emphasized (Whelan. 1966). *Pseudomonas* isolamylase which was newly found by Harada et al., (1968) will offer an useful tool for structural analysis of the polysaccharide, because it is easily prepared and the specificity of purified enzyme is quite reliable (Yokobayashi et al., 1969).

ACKNOWLEDGMENTS

The authors are grateful to Professor T. Harada, Laboratory of Applied Biochemistry, Institute of Scientific and Industrial Research of Osaka University, who made these studies possible. They also thank Mr. K. Yokobayashi who kindly supplied a specimen of isoamylase he isolated, and other stuff of the laboratory of Applied Biochemistry of this Institute for their friendly help and numerous suggustions during the course of this work. They are also indebted to Dr. Y. Hirachi, Department of Microbiology, Osaka University Dental School, for a gift of L-11 enzyme.

REFERENCES

Abdullah, M., B. J. Catley, E. Y. C. Lee, J. Robyt, K. Wallenfels and W. J. Wehlan. 1966. The mechanism of carbohydrase action. II. Pullulanase, an enzyme specific for the hydrolysis of α-1,6-bonds in amylaseose oligo- and polysaccharides. Cereal Chem. 43 : 111.

Aarchibald, A. R., I. D. Fleming, A. M. Liddle, D. J. Manners, G. A. Mercer and A. Wright. 1961. α-1,4-glucosans. Part XI. The absorption spectra of glycogen- and amylopectin-iodine complexes. J. Chem. Soc. 1183–1190.

Ashwell, G. 1957. Colorimetric analysis of sugars. p. 73. *In* S. P. Colowick and N. O. Kaplan [ed.]. Method in Enzymology. VIII. Academic Press,

New York and London.
Dawes, E. A. and D. W. Ribbons. 1964. Some aspects of the endogenous metabolism of bacteria. Bacteriol. Rev. 28 : 126–149.
Fleury, P. and J. Lange. 1933. Determination of periodic acid in the presence of iodic acid. J. Pharm. Chem. 17 : 107.
Ghuysen, J. M., D. J. Tipper and J. L. Stromінger. 1966. Enzymes that degrade bacterial cell walls. p. 685 to 699. *In* D. F. Neufeld and V. Ginsburg [ed.]. Method in Enzymology VIII. Academic Press, New York and London.
Gorin, P. A. and J. F. T. Spencer. 1968. Structural chemistry of fungal polysaccharides. p. 367 to 376. *In* M. L. Wolfrom and R. S. Tipson [ed.]. Advance in Carbohydrate Chemistry XXIII. Academic Press, New York and London.
Hakomori, S. 1964. A rapid permethylation of glycolipid and polysaccharide catalized by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. 55 : 205–208.
Hamilton, J. K. and Smity, F. 1956. Reduction of the products of periodate oxidation of carbohydrate. II. A new method for the end-group assay of amylopectin. J. Amer. Chem. Soc. 18 : 5907–5909.
Harada, T., K. Yokobayashi and A. Misaki. 1968. Formation of isoamylase by *Pseudomonas*. Appl. Microbiol. 16 : 1439–1444.
Holme, T. and H. Palmistierna. 1956. On the glycogen in *Escherichia coli* B. its synthesis and breakdown and its specific labelling with ^{14}C. Acta Chem. Scand. 10 : 1557–1562.
Kato, K., S. Kotani, T. Matsubara, T. Kogami, S. Hashimoto, M. Chimori and I. Kazekawa. 1962. Lysis of *Staphylococcus aureus* cell walls by lytic enzyme purified from culture supernatants of *Flavobacterium species*. Biken J. 5 : 155–179.
Kato, K., T. Hirata, Y. Murayama, H. Suginaka and S. Kotani. 1968. Studies on the mode of action of *Flavobacterium* L-11 enzyme on the cell walls of *Staphylococcus aureus* strain Copenhagen. Identification of isolated cell wall peptide. Biken J. 11 : 1–12.
Kotani, S., K. Kato, T. Matsubara, T. Hirano and M. Higashigawa. 1959. Staphylolytic activity of a culture filtrate of a *Flavobacterium* species. Isolation from soil. Biken's J. 2 : 211–213.
Kolenbrander, P. E. and J. C. Ensign. 1968. Isolation and chemical structure of the peptidoglycan of *Spirillum serpens* cell walls. J. Bacteriol. 95 : 201–210.
Leutgeb, W. and W. Weidel. 1963. Über ein Coli-Zellwandpräparaten zurückgehaltenes Glycogen. Z. Naturforsch. 18b : 1060–1062.
Leutgeb, W., D. Maass and W. Weidel. 1963. Herstellung und quantitative Zusammensetzung protein- und glucosefreier Stüzmembranen (R-layers) aus Coli-Zellwänden. Z. Naturforsch. 18b : 1062–1064.
Manners, D. J. 1957. The molecular structure of glycogens. p. 261 to 298. *In* M. L. Wolfrom and R. S. Tipson [ed.]. Advance in Carbohydrate Chemistry. Academic Press, New York and London.
Misaki, A., J. Johnson, Jr., S. Kirkwood, J. V. Scaletti and F. Smith. 1968. Structure of the cell wall glucan of yeast. Carbohyd. Res. 6 : 150–164.
Misaki, A. and S. Yukawa. 1966. Studies on cell walls of Mycobacteria. II. Constitution of polysaccharide from BCG cell walls. J. Biochem. 59 : 511–520.
Park, J. T. and M. J. Johnson. 1949. A submicrodetermination of glucose. J. Biol. Chem. 181 : 149–151.
Roseman, S. and I. Daffiner. 1956. Colorimetric method for determinations of glucosamine and galactosamine. Anal. Chem. 28 : 1743–1746.
Sandford, P. A. and H. E. Conrad. 1966. The structure of the *Aerobacter aerogenes* A3(S1) polysacchride. I. A reexamination using improved procedures for methylation analysis. Biochemistry. 5 : 1508–1517.
Sawardeker, J. S., H. H. Sloneker and A. Jeanls. 1965. Quantitative determination of monosacchrides as their alditol acetate by gas liquid chromatography. Anal. Chem. 37 : 1602–1604.
Stanier, R. Y., M. Doudoroff, R. Kunisawa and R. Contopolilou. 1959. The role of organic substrates in bacterial photosynthesis. Proc. Natl. Acad. Sci. U.S. 45 : 1246–1260.
Sweeley, C. C., R. Bently, M. Makita and W. W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugar and related substances. J. Amer. Chem. Soc. 85 : 2497–2508.
Takikawa, I. 1958. Studies on pathogenic halophilic bacteria. Yokohama Med. Bull. 9 : 313–322.
Tamura, T. 1969. Studies on the cell envelope of

Vibrio parahaemolyticus with special reference to hypotonic lysis of the cells (in Japanese). Med. J. Osaka Univ. 21 : 129–138.

Tamura, T., T. Fujino, K. Kondo and S. Kotani. 1968. Occurence of poly-β-hydroxybutyric acid inclusions in *Vibrio parahaemolyticus* A55. Biken J. 11 : 225–234.

Tanaka, S., T. Fujita and H. Hagihira. 1969. Reguration of amylase synthesis by *Vibrio parahaemolyticus*. Biken J. 12 : 119–124.

Toama, M. A. and K. B. Raper. 1967. Mycrocysts of the cellular slime mold *Polysphondylium pallidum*. II. Chemistry of the microcyst walls. J. Bacteriol. 94 : 1150–1153.

Ward. C. and B. E., Wright. 1965. Cell wall synthesis in *Dictyostelium discoideum*. I. *In vitro* symthesis from uridine diphosphoglucose. Biochemistry. 4 : 2021–2027.

Weidel, W., H. Frank and W. Leutgeb. 1963. Autolytic enzymes as a source of error in the preparation and study of gram-negative cell walls. J. Gen. Microbiol. 30 : 127–130.

Whelan, W. J. 1966. The contribution of enzymes to the structural analysis of glycogen and starch. Biochem. J. 100 : 1 p.

Yokobayashi, Y., A. Misaki and T. Harada. 1969. Specificity of *Pseudomonas* isoamylase. Agr. Biol. Chem., 33 : 625–627.