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STUDIES OF THE CELL ENVELOPE OF *VIBRIO PARAHAEMOLYTICUS* A55: ISOLATION AND PURIFICATION OF BAG-SHAPED PEPTIDOGLYCAN (MUREIN SACCULUS)[1]

TOSHIHIDE TAMURA

Department of Tuberculosis Research II, Research Institute for Micorobial Diseases, Osaka University, Yamada-Kami, Suita, Osaka

KEIJIRO KATO, SADAKO IWATA and SHOZO KOTANI

Department of Microbiology, Osaka University Dental School, Joan-Cho, Kita-Ku, Osaka

TOSHIYUKI KITAURA

Laboratory of Microbiology, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-Ku, Osaka

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SUMMARY The peptidoglycan (PG) component of the envelope of *Vibrio parahaemolyticus* A55 was studied in relation to the salt dependency of the organism. A bag-shaped PG (murein sacculus) was isolated and purified by digestion of freshly harvested intact whole cells with sodium dodecyl sulfate (SDS) and then protease treatment. Whole cells lyze in salt-deficient or hypotonic conditions and this can be measured by turbidometry. Lysis is prevented by addition of 0.35 M NaCl or acidification to below pH 4.5. The isolated PG or murein sacculi did not seem to require salt for structural integrity. The PG obtained by the same method from subcellular fractions, that is, envelope fractions, prepared either with or without NaCl, was extensively fragmented. The main amino acids and amino sugars of the murein sacculus were glucosamine, muramic acid, alanine, glutamic acid, 2, 6-diamnopimelic acid (A_2pm) in a molar ratio of 0.4:0.6:1.7:0.9:1.0. A polyglucose, which was only degraded by *Pseudomonas* isoamylase, and poly-β-hydroxybutyrate (PHBA) were found in the murein sacculus. The murein sacculus of the organism is discussed in comparison with PG of other gram negative bacteria and marine or halophilic microorganisms.

1 Parts of this work were presented at the 40th Annual Meeting of the Japanese Society of Microbiology in Nagoya in March, 1967 and at the 21st Kansai Area Meeting of the Japanese Society of Microbiology in Osaka in September, 1968.

INTRODUCTION

Vibrio parahaemolyticus is a halophilic marine bacterium found in water of coastal areas and estuaries and it requires appropriate amounts of salts. It is unique in that it can also proliferate in mammals, producing strong entero- and cardiotoxic substances that are occasionally fatal to human beings. (Miwatani and Takeda, 1976). Deneke and Colwell (1973) suggested that it my represent a biological intermediate between marine and terrestrial microorganisms. We became interested in whether the cell envelope of *V. parahaemolyticus* has any unique structure which fits the organism for both marine and parasitic environments.

The main constituents of gram negative bacteria are outer-, inner- and PG-layers. The shape of the envelope is due to a rigid layer consisting of a complex of PG or murein sacculus and lipoprotein (Martin and Frank, 1962). PG works also as a crucial site for bacterial cell growth and division (Ryter et al., 1973). The endotoxin-like activity of PG has become attractive recently because of its clinical significance as an immunopotentiator (Kotani et al., 1975; Heymer, 1975).

The chemical structure of PG is sometimes associated with physiological and ecological characteristics of certain bacteria. For instance, in *Arthrobacter* (Krulwich et al., 1967), *Myxobacter* (Johnson and White, 1972) and *Caulobacter* (Goodwin and Shedlarski, 1975), the structure of PG alters during morphogenesis of the organisms. Schleifer and Kandler (1972) have reviewed the taxonomical and ecological significances of the variations in the structure in different groups of microorganisms.

Since the time of its second discovery made by Takikawa (1958), *V. parahaemolyticus* has been known to be inactivated very rapidly in distilled water (Lee, 1972). Kitaura et al. (1965) noticed that cells or subcellular fractions prepared in 3% NaCl solution became much smaller when washed with distilled water. The envelopes of some marine or halophilic bacteria were solubilized (Buckmire and MacLeod, 1965) or fragmented (Ohtani and Masui, 1969) when suspended in low salt solution. Constituents of the envelopes in these organisms seemed to have salt-dependent structure. Particularly, it was considered that the PG layer of the organisms was not continuous but that it consisted of units of PG patches assembling each other by the presence of cation. Thus as a first step in studies on the envelope of the organism, we examined whether the structure of PG, which is the central part of the envelope, is salt-dependent. For this purpose, we examined lysis of the organism in solution of low salt concentration and then we attempted to demonstrate morphological appearence of chemically purified PG component.

MATERIALS AND METHODS

1. *Organism and cultivation*

The organism used throughout was *Vibrio parahaemolyticus* (strain A55, serotypes 05: K15, Kanagawa phenomenon-hemolysin production positive). The strain was originally isolated by Takikawa as a causative agent during an epidemic of food poisoning in Yokohama, Japan, in 1955 (Takikawa, 1958) and has been maintained at Osaka City Institute of Hygiene.

Part of the methods used to maintain the culture and to obtain large populations of cells have been described (Tamura et al., 1969). The standard medium was composed of 1% each of casamino acids, yeast extract and maltose and 0.5 M NaCl, adjusted to pH 7.8 with NaOH. The strain grew very rapidly in this medium with a generation time of about 10 min under vigorous reciprocal agitation at 37 C and it reached the late exponential growth phase in four to five hours. At this stage a large amount of crushed ice was added and the cells were collected by centrifugation at $11,300 \times g$ for 30 min in the cold. The cells were washed three times with cold buffered saline (BS) consisting of 0.02 M potassium phosphate (pH 7.0) and 0.5 M NaCl. To obtaining intact murein sacculi, fresh cells were rapidly treated with sodium dodecylsulfate (SDS) but

for other purposes they were frozen until required. Before lyophilization, cells or cell envelopes were washed with a small amount of distrilled water, adjusted to pH 4.5 with a few drops of acetic acid.

2. *Measurement of cell lysis*

A cell suspension was adjusted to an optical density at 550 nm (OD_{550}) of 10.0 with BS and then diluted 20 times with various test solutions to give a final volume of 2.0 ml. The resulting decrease in OD was measured in a Shimadzu Bausch and Lomb Spectronic 20 colorimeter. For this experiment, cells were obtained from an exponentially growing culture and used within 24 hr. For measurement of release of materials with ultraviolet (UV) absorption from the cells during lysis, lysis was interrupted at the desired time by adding an equal volume of 0.02 M potassium phosphate buffer (pH 7.0) containing 1 M NaCl to the cell suspension. Then the mixture was centrifuged at $11,500 \times g$ for 30 min and the OD of the supernatant at 260 nm was measured. Lysis was expressed as the reduction in OD as a percentage of the initial OD of control cells in BS.

The cell counts were made before and after lysis in a Petroff-Hauser cell counter under a phase contrast microscope.

3. *Preparation of cell envelope fraction*

Two alternative methods were employed to prepare cell envelopes. One was conventional mechanical disruption of the cells and then differential centrifugation. The other was hypotonic treatment of the cells, based on the assumption that the PG component in the cell envelope was not released appreciably after lysis of the cells in solutions of low salt concentration (see Results, section 2).

1) Preparation of cell envelopes by mechanical treatment in the presence of 0.5 M NaCl (envelopes-I)

A paste of frozen intact cells, 22 g wet weight, was suspended in 150 ml of BS and shaken (2,000 strokes per min) in a Braun cell homogenizer for 10 min with 30 g of glass beads (Glasperlen, 0.11 to 0.12 mm) in a Duran glass bottle of 75 ml volume with CO_2 cooling. The disrupted cell suspension was diluted 10 fold with BS and the envelope fraction was collected by differential centrifugation at 4,500 $\times g$ and $10,500 \times g$ for 30 min each. This fraction was washed twice with 500 ml of BS with centrifugation, and then treated with 500 ml of 1 M NaCl and washed 3 times with 500 ml volumes of BS. The yield of the envelope fraction (envelopes-I) was 9% by weight of the whole cells used as starting material (Table 1).

2) Preparation of cell envelopes by hypotonic treatment (envelopes-II)

A frozen paste of intact cells, 12 g wet weight, was suspended in 1.2 liter of chilled 0.02 M potassium phosphate buffer, pH 7.0 and stirred for 1 hr at 4 C. The lysed cells were collected by centrifugation at $11,300 \times g$ for 30 min and washed successively once with 500 ml of 1 M NaCl solution and three times with distilled water with centrifugation. The lysed cells were dispersed in 100 ml of the above buffer containing 10 mg of crystalline trypsin (Sigma), incubated at 37 C for 1 hr and centrifuged as above. Trypsin digestion was repeated and then the cells were washed thoroughly with distilled water and lyophilized. The yield of this preparation was 11% by weight of the initial whole cells (Table 1). The supernatant and washing obtained after lysis and trypsinization were pooled, dialyzed against distilled water and lyophilized for weighing and analysis of diaminopimelic acid (A_2pm). After this treatment the cells retained a rod-shape but seemed to be free from cytoplasmic debris other than granular inclusions (Fig. 6). As seen in Table 1, the A_2pm contents of envelopes-I and -II were almost equal.

4. *Enzymatic digestions*

L-11 enzyme: the enzyme was obtained from *Flavobacterium* species and was shown to lyse PG due to its endopeptidase activities hydrolyzing -D-Ala-Gly-D-Ala-L-Ala-, or -Gly-Gly- linkages and/or *N*-acetylmuramyl-L-alanine amidase (Kato et al., 1962; Kato and Strominger, 1968; Kato et al., 1968; Matsuda, Kotani and Kato, 1968; Suginaka et al., 1970; Hirata, 1970; Hamada et al., 1971; Hirachi et al., 1971). Five mg of PG (step 3 or 5 preparation, see Results, section 5) were hydrolysed at 37 C in 2 ml of 0.0125 M phosphate buffer of pH 8.0 containing 12 units (against *Staphylococcus aureus* cell walls) of the enzyme. L-3 enzyme: the main activities of the enzyme were D-Ala-*meso*-A_2pm endopeptidase and *N*-acetylmuramyl-L-alanine amidase, and the enzyme was partially, purified from the culture filtrate of *Streptomyces* species (Mori et al., 1960; Mori and Kotani, 1962; Kato, Strominger and Kotani, 1968; Matsuda, Kotani and Kato, 1968; Katayama, 1973). The PG pre-

paration (500 μg) was incubated at 37 C in 200 μl of 0.02 M phosphate buffer of pH 7.8 containing 8 units (against *Lactobacillus plantarum* cells) of the enzyme. ALE enzyme: the enzyme was produced by *Staphylococcus epidermidis* strain EP-K1 (Suginaka et al., 1967). The enzyme showed *N*-acetylmuramyl-L-alanine amidase and -Gly-Gly- endopeptidase activities. The PG preparation (500 μg) was incubated at 37 C with one unit (against *Staphylococcus aureus* cell walls) of the enzyme in 200 μl of 0.0125 M phosphate buffer of pH 6.8 containing 0.1 M NaCl. The hydrolyse of PG by these three enzymes were monitored by measuring increase of free amino groups. Lysozyme (Sigma, twice crystallized): PG was hydrolyzed at 37 C in 1 ml of 0.1 M Tris-HCl buffer, pH 8.5 containing 1 mg of PG and 20 μg of the enzyme. *Charalopsis* enzyme: this enzyme was a generous gift from Dr. P. A. Miller (Lederle Lab., American Cyanamid Co., N.Y.). The PG preparation (5 mg) was incubated at 37 C in 0.025 M acetate buffer, pH 4.5 containing 50 μg of the enzyme for 4 hr. The hydrolytic actions of lysozyme and the *Charalopsis* enzyme were monitored by measuring increase in reducing power. Amylases: *Pseudomonas* isoamylase was kindly supplied by Prof. T. Harada of the Institute of Scientific and Industrial Research, Osaka University. The enzyme was shown to hydrolyze β-1, 6 glucosidic interchain linkages in amylopectin and glycogen (Harada et al., 1968). Crystalline α- and β-amylases were purchased from Sigma Chemical Co. Details of the actions of amylases have been described (Tamura et al., 1969). For large scale digestions, the amount of each constituent in the reaction mixtures were increased appropriately.

5. *Amino acid and amino sugar analyses*

Amino acids and amino sugars were analyzed in a Yanagimoto Type LC-5 Amino Acid Autoanalyzer after hydrolyzing samples in 6 N HCl for 12 hr at 100 C in sealed tubes. No correction was made for loss of hexosamines during acid hydrolysis. NH_2 terminal amino groups and total amino groups were determined by thin layer chromatography using the procedure of Ghuysen et al. (1966) as modified by Matsuda et al. (1968). A_2pm was estimated by paper chromatography by the method of Brown et al. (1962).

6. *Carbohydrate analyses*

Total hexosamine was determined by a modification of the Morgan-Elson method. Hexose was measured with anthrone reagent (Ashwell, 1957). Reducing power was measured by the method of Park and Johnson (1949). Glucose was estimated either by an enzymatic method using Glucostat (Worthington Biochemical Co.) or by gas chromatography as described by Sweeley et al. (1963). Polyglucose was measured as described previously (Tamura et al., 1969).

7. *Other chemical analyses*

Phosphorus was assayed by the method of Lowry et al. (1954). Poly-β-hydroxybutyrate (PHBA) was assayed by the method of Law and Slepecky (1961). Total protein was determined by the Follin phenol method of Lowry et al. (1951).

8. *Gel filtration*

Columns of Sephadex G-100, G-75, G-50 and G-25 (Pharmacia Fine Chemicals, Inc.) were employed for gel filtration of enzymatically digested preparations. All columns were equilibrated with water and developed at 4 C.

9. *Electron microscopy*

Preparations to be shadowed were placed on Formvar coated grids and shadowed at an angle of 25° to 30° with plutinum. Thin sectioned preparations were made essentially according to the method of Murray et al. (1965). Intact cells of *V. parahaemolyticus* was prefixed in 0.5% glutaraldehyde in 0.02 M phosphate buffer, pH 6.2 containing 0.5 M NaCl. All preparations were photographed with a type 120 electronmicroscope (Japan Electron Optics Laboratory Co.).

RESULTS

1. *Turbidity change of cell suspensions*

As shown in Fig. 1, when cells were exposed to solution of low salt concentration by diluting the cell suspension with 0.02 M phosphate buffer of pH 7.0 (final NaCl concentration, 25 mM), as described in the Materials and Methods, the turbidity or optical density at 550 nm (OD_{550}) decreased almost 50% within a minute even in ice-cold conditions. The initial rapid OD decrease was followed by a slower OD decrease. The initial rapid OD decrease appeared to be temperature independ-

ent but the subsequent decrease was remarkably accelerated by raising the incubation temperature. A total OD decrease of 80% was observed on prolonged incubation. When the cells were suspended in a series of decreasing concentrations of NaCl (Fig. 2), the observed decrease in turbidity was roughly inversely proportional to the NaCl concentration. This descrease in turbidity was accompanied by release of UV-absorbing materials into the medium. The steep bent in the plot of OD_{550} versus NaCl concentration between 0.3 to 0.2 M NaCl may indicate a critical NaCl concentration below which release of UV-absorbing materials occurred. The results show that an NaCl concentration of at least 0.35 M is required to stabilize the cells against change in turbidity. LiCl prevented lysis as effectively as NaCl, and KCl was slightly less effective (Fig. 2). Divalent cations such as Mg^{++} and Ca^{++} were effective at much lower concentrations than monovalent cations (Fig. 3). Lysis was prevented by fixing the cells in 2% formalin for 16 hr or boiling them in 0.5 M NaCl solution, but not by treatment with various enzyme inhibitors, such as 100 μg/ml of soy bean trypsin inhibitor, 1% diisopropylfluorophosphate or 10 mM parachloromercuribenzoate. Lysis was also prevented by acidification, even at low cation concentration. As shown in Fig. 4, 0.02 M acetate buffer, pH 4.5 prevented both decrease in turbidity and release of UV-absorbing materials. The OD increase below pH 4.5 seemed to be due to aggregation of the cells. Lysis was greater at alkaline pH. Values more than 95% decrease in turbidity was observed at pH 11 to 12. The critical pH seemed to be between pH 5.0 and 6.0 and above this release of UV-absorbing materials was accelerated. A hypotonic solution containing only non-ionic components, such as 0.5 M sucrose or 10% carbowax 4000, did not prevent lysis appreciably and further addition of NaCl was necessary to prevent lysis completely. Spheroplasts made with 1,000

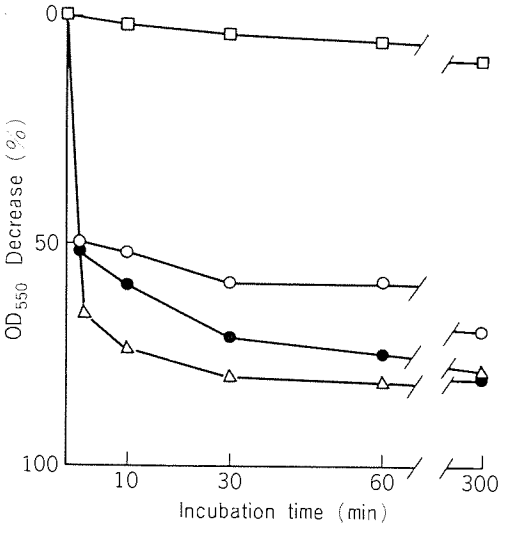

FIGURE 1. Lysis of *V. parahaemolyticus* A55 in 0.02 M phosphate buffer, pH 7.0. Cells suspended in BS at room temperature (□——□), in 0.02 M phosphate buffer of pH 7.0 in an ice bath (○——○), in the same buffer at room temperature (●——●), in the same buffer at 37 C (△——△).

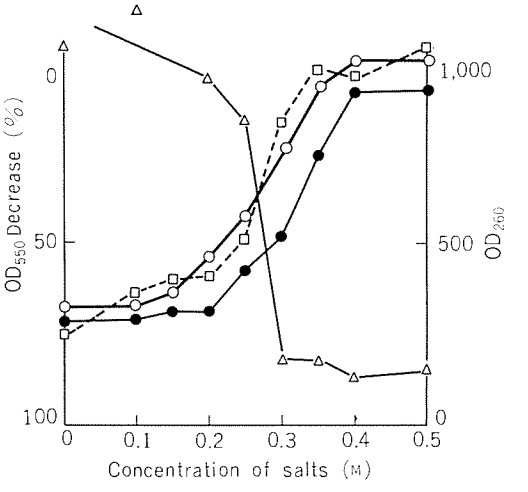

FIGURE 2. Relation of concentration of salt of monovalent cations with lysis of *V. parahaemolyticus* A55 and concomitant release of UV-absorbing materials. The OD at 550 nm was measured 60 min after suspending the cells in 0.02 M phosphate buffer, pH 7.2 containing various concentrations of salts at room temperature. OD_{550} reduction in NaCl (○——○), KCl (●——●) and LiCl (□——□). Release of UV-absorbing materials in NaCl solution (△——△).

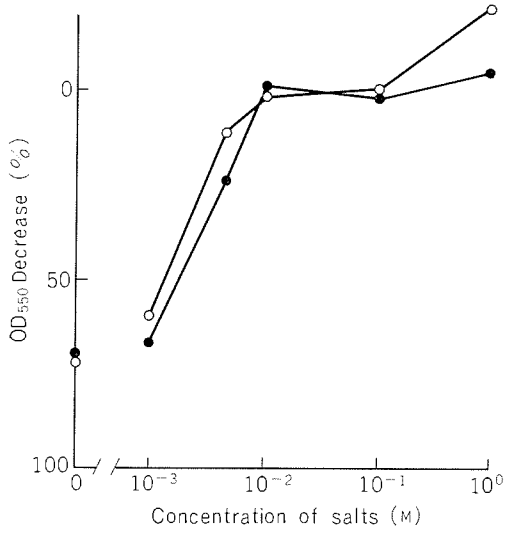

FIGURE 3. Relation of concentration of salts of divalent cations and lysis of *V. parahaemolyticus* A55. The OD at 550 nm was measured 60 min after suspending the cells in salt solution in 0.02 M phosophate buffer, pH 7.0 at room temperature. $MgCl_2$ (○——○), $CaCl_2$ (●——●).

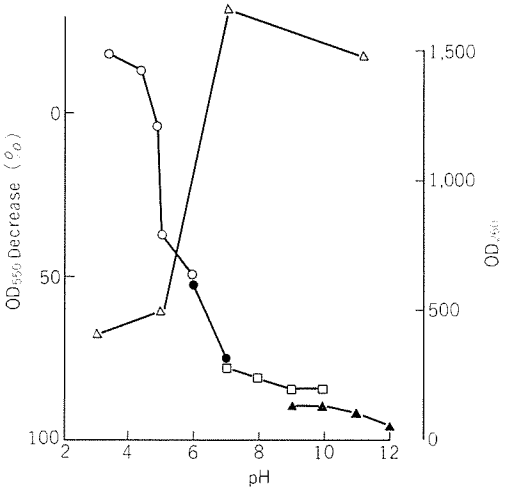

FIGURE 4. Relation between the pH of the suspension medium and lysis of *V. parahaemolyticus* A55. The OD_{550} nm was measured 60 min after suspending the cells in 0.02 M buffer of various pH values at room temperature. OD_{550} reduction in acetate buffer (○——○), phosphate buffer (●——●), Tris-HCl buffer (□——□) and glycine-NaOH buffer (▲——▲). Release of UV-absorbing materials (△——△).

units/ml of Penicillin G also required NaCl as a stabilizer in addition to sucrose.

2. *Effect of low salt concentration on cell envelopes-I*

When cell envelopes-I, prepared by mechanical disruption in 0.5 M NaCl, were suspended in solution of low salt concentration, they showed 30% loss of turbidity within 30 min. As with whole cells, most of the OD decrease of a suspension of the envelopes occurred within one minute and so seemed to be temperature independent. However, the effect of NaCl concentration on lysis of cell envelopes was different, since, as seen in Fig. 5, the concentration of NaCl needed to prevent lysis of cell envelopes-I was about half that needed to prevent lysis of whole cell. The envelopes-I were lyzed in low salt solution as described above and centrifuged at 11,500 × *g* for 30 min. The soluble materials recovered in the supernatant, consisted 25% dry weight of the starting envelopes-I (Table 1). Paper chromatographic analysis of an acid hydrolyzate of these solubilized materials showed that only about 2% of the total A_2pm residues were released from the envelopes-I. Thus the most of the

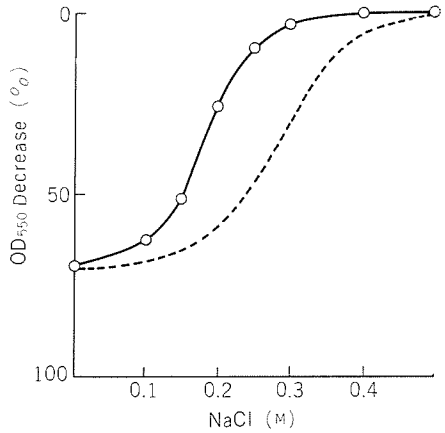

FIGURE 5. Relation between the concentration of NaCl in 0.02 M phosphate buffer, pH 7.0 and lysis of cell envelopes-I of *V. parahaemolyticus* A55 (○——○). Experimental conditions were as described for Fig. 2. Data on lysis of whole cells are take from Fig. 2 (……).

TABLE 1. *Yield and A_2pm content of envelopes-I and envelopes-II with their subfractions obtained with lysis or trypsin digestion*

Preparation	Yield		A_2pm[a] nmoles/mg
	g	%	
Starting whole cells	22	100	ND
Envelopes-I	1.9	9	34
Envelopes-I after lysis			
Released	0.5	2.3	2.6
Residue	1.0	4.5	48
Starting whole cell	12	100	ND[b]
Lysed whole cell residue	2.2	18	26
Trypsinized lysed whole cell			
Released	0.8	6.7	5.5
Residue (envelopes-II)	1.3	11	42

[a] Samples of 5 to 10 mg were hydrolyzed in 6 N HCl for 12 hr and A_2pm was separated by paper chromatography by the method of Brown et al. (1962).

[b] Not determined.

PG component in the envelopes-I was not liberated by lysis, even though one quarter of the cell envelope constituents became soluble.

3. *Morphological observations on cell lysis*

Under a light or phase contrast microscope, individual cells were seen to stain weakly with basic fuchsin and their refractivity decreased during lysis. However, the cells retained their rod-shaped contour and were almost the same size as intact cells. Moreover, the total number of cells, estimated in a Petroff-Hauser cell counter, was the same before and after lysis in solution of low salt concentration at neutral pH. Thus the decrease in turbidity was not due to either destruction of the cells or decrease in the cell number, but judging from the decrease in refractivity of individual cells, it may have been due to leakage of cytoplasmic materials.

Cells in a strongly alkaline solution of low salt concentration behaved in a somewhat different way. Namely, when cells were suspended in ice cold 0.02 M glycine-NaOH buffer, pH 12, and then rapidly neutralized with HCl and examined under a phase contrast microscope, cells with a rod-shaped contour could no longer be seen but only small fragments of cells. This phenomenon was not studied further.

Electronmicroscopic examination confirmed these findings on lysis of cells in solution of low NaCl concentration. Figure 6 shows the appearence of a shadowed preparation of envelopes-II prepared by hypotonic treatment of

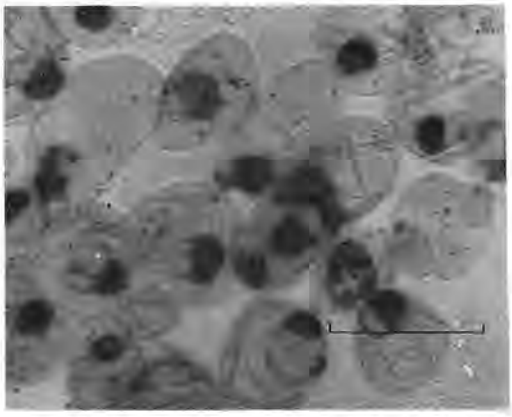

FIGURE 6. Shadowed preparation of envelopes-II of *V. parahaemolyticus* A55. Rod-shaped or round, smooth surfaced membranous structures with eletron dense inclusions can be seen. Scale: 1 μm.

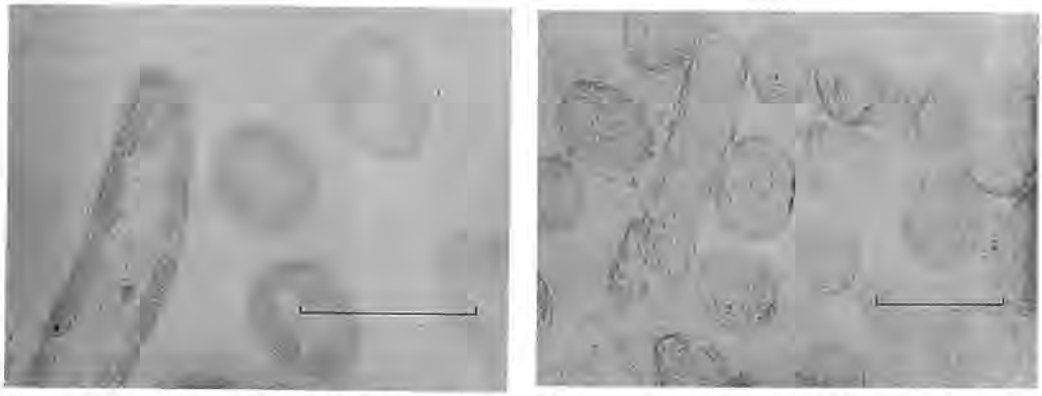

FIGURE 7A and 7B. Section of *V. parahaemolyticus* A55 prepared by the method of Murray et al. (1965).

7A. Intact whole cells prefixed in the presence of 0.5 M NaCl. The envelope, consisting of a complex of outer and inner membranes, can be seen. The displaced portion in the cytoplasm may be a mass of nuclear material surrounded by ribonucleoproteins. PHBA inclusions cannot be seen in the preparation because for this particular specimen, cells were grown in maltose-free medium in which the cells do not accumulate PHBA. Scale: 1 μm.

7B. Lyzed cells prefixed without NaCl. The cells were allowed to lyse for 60 min at room temperture in 0.02 M phosphate buffer, pH 7.0 as described in the text and prefixed in the same buffer. Note that the rod shape is still retained. The outer and inner layers are separated. Blebs or protrusions from the outer layer can be seen. The boundary of the nuclear material has disappeared and ribonucleoprotein particles are distributed throughout the cytoplasmic space. Scale: 1 μm.

whole cells as described in Materials and Methods: the rod-shaped membranous structures with electron dense inclusions can be seen. Thin sections of intact cells (Fig. 7A) fixed in the presence of 0.5 M NaCl showed that the cells had the typical double membraned envelope profile of a gram negative organism at the periphery. The PG layer, seen in other gram negative bacteria as an electron dense single layer between the outer and inner layers when stained with uranium acetate (Murray et al., 1965) was not detectable. However the inner layer stained more strongly and appeared thicker than the outer layer. After lysis, cells had the same two membranes but they were much looser and were separated from each other in many places (Fig. 7B). The inner layer seemed to be damaged much more than the outer layer during lysis.

4. *Attempt to isolate PG from envelopes-I and -II*

The amounts of A_2pm in the various fractions shown in Table 1 indicate that most of the PG components were recovered in the envelopes irrespective of whether they were prepared with or without NaCl. Accordingly attempts were made to isolate the PG fraction from envelopes-I and -II by SDS digestion as described in section 5. The insoluble residues of envelopes-I after digestion with SDS and proteases were submitted to chemical analysis (Table 2). A_2pm, alanine, glutamic acid and hexosamine were present in a ratio of approximately 1: 2: 2: 1. Hexose was also found but PHBA was not detected. The relatively small amount of hexosamine was probably due to its destruction by acid hydrolysis. If the PG of the organism is similar to that of *E. coli*, then the real total contribution of PG in the whole cells was probably much less than 0.5%, calculated from the amount of amino acids and hexosamine shown in Table 2. This value is much lower than that in *E. coli* (White et al., 1968). In an electronmicrograph of a shadowed preparation, the PG obtained from envelopes-I was seen as irregular-shaped frag-

TABLE 2. *Yield and chemical copmosition of peptidoglycan preparations obtained from envelopes-I and envelopes-II*

	PG from envelopes-I		PG from envelopes-II	
	(g)	(%)	(g)	(%)
Starting material (envelopes-I or -II)	1.0	100	1.0	100
Peptidoglycan obtained	0.087	8.7	0.036	3.6
Per cent yield of peptidoglycan from whole cell[a]		0.76		0.67
	(nmoles/mg)	(%)	(nmoles/mg)	(%)
Total NH_2 groups[b]	2105	32	3623	54
Hexose[c]	1390	25	890	16
PHBA[d]	0		0	
	(nmoles/mg)	(Molar ratio)	(nmoles/mg)	(Molar ratio)
A_2pm[e]	251	1.0	383	1.0
Alanine[e]	587	2.3	885	2.3
Glutamic acid[e]	424	1.7	322	0.8
Hexosamine[f]	295	1.2	475	1.2

[a] Calculated from the data in Table 1.
[b] Assayed by thin layer chromatography of a dinitrophenylated acid hydrolyzate using glutamic acid as a standard (Matsuda et al., 1968).
[c] Assayed by the anthrone method.
[d] Assayed by the method of Law and Slepecky (1961).
[e] A dinitrophenylated sample was hydrolyzed with 6 N HCl for 12 hr and assayed by thin layer chromatography.
[f] Assayed by the Morgan-Elson method.

FIGURE 8. Shadowed preparation of the peptidoglycan isolated from envelopes-I of *V. parahaemolyticus* A55 by SDS treatment. Peptidoglycan is irregularly fragmented. Scale: 1 μm.

ments and the original rod-shape was not discernible (Fig. 8). PG fractions from envelopes-II appeared similar to those from envelopes-I both chemically and morphologically. Since entirely intact PG was not necessarily essential for maintenance of the rod shapes as in the case of *Halobacterium* sp. and *Myxococcus xanthus* (White et al., 1968), so *V. parahaemolyticus* may also possess an incomplete type of PG. Another possible explanation for why the PG of the organism was incomplete is that PG may have been partially autolyzed in the course of manipulations of the cells. In consistency with this possibility, Brown et al. (1968) suggested that hypotonic lysis as well as mechanical treatment acted as a trigger to activate hidden autolytic enzymes in the cell envelopes.

A reviced method was deviced involving rapid SDS digestion of fresh whole cells in the presence of enough salt and dispension with mechanical treatment. The method should reduce the chance of activation of autolytic enzymes.

5. *Isolation of PG by direct treatment of intact whole cells with SDS*

1) Step 1 (SDS treatment). Hundred gram wet weight of freshly harvested cells were suspended in 500 ml of chilled 0.02 M potassium phosphate buffer containing 0.5 M NaCl and 0.01 M $MgCl_2$ and rapidly mixed with equal volume of boiling 2% SDS solution containing 0.5 M NaCl and 0.01 M $MgCl_2$ and shaken vigorously by hand for a few minutes. The mixture was cooled to room temperature, and then 1 mg of crystalline deoxyribonuclease (Sigma) was added to reduce the viscosity and the mixture was stirred with a magnetic stirrer for 3 hr at room temperature. The mixture was diluted two fold with water and subjected to ultracentrifugation of $58,000 \times g$ for 60 min at room temperature. SDS solution must be used at room temperature because SDS is very insoluble in water in the cold. The dark brown supernatant obtained by centrifugation was discarded and the pale brown pellet was resuspended in 1% SDS, stirred for 1 hr and centrifuged as before. The insoluble pellet was washed with 6 M urea with centrifugation until no SDS could be detected in either the washings or residue. The presence of SDS was tested by seeing whether a white flocculus formed when a few drops of saturated $Ba(OH)_2$ were added. Before the final washing, the prepration was centrifuged at low speed ($1,500 \times g$ for 30 min) to remove insoluble particulate contaminants. The loose, cream-colored precipitate obtained after the final washing corresponded to about 5% dry weight of the starting intact whole cells. This preparation was seen to consist of rod shaped membranous structure with a rough granular surface by electron-microscopy and analysis showed that it contained amino acids in excess of PG (Table 4) and also hexose and PHBA (Table 3).

Analytical grade of SDS and urea were purchased from Nakarai Chemicals. Fresh urea solution was prepared each time just before use since it is said that urea solution on storage develope ammonium cyanate which react with amino and sulphydryl groups of protein and occationally inactivate its biological function. It was recommended, therefore, that urea solution should be deionized by ion exchange column chromatography to remove cyanate completely (Ito and Ishihama, 1973). We used non-deionized fresh urea solution throughout the study. Although cyanate in fresh urea solution might be very small, the influence of cyanate on PG during preparation

TABLE 3. *Chemical composition of the preparation of murein sacculi of V. parahaemolyticus A55 at each purification step*

Constituents	Preparation of murein sacculus at			
	Step 1 (%)	Step 2 (%)	Step 3 (%)	Step 5 (%)
Amino acids and amino sugars[a]	57.5	41.5	35.5	43.1
Hexose[b]	18.6	54.0	66.0	0.9
PHBA[c]	4.0	7.5	11.0	0.4

[a] Calculated from data obtained with an amino acid autoanalayzer.

[b] Estimated with anthrone except Step 5 preparation. The Step 5 preparation was assayed by the Glucostat method.

[c] Estimated by the method of Law and Slepecky (1961).

remains in question.

2) Step 2 (digestion with trypsin and pepsin). The preparation from Step 1 was suspended in 100 ml of 0.02 M Tris HCl buffer, pH 7.0 containing 10 mg of crystalline trypsin (Sigma) and incubated with gentle shaking for 4 hr at 37 C. Then it was subjected to ultracentrifugation and the precipitate was digested with trypsin once more in the same manner. The residue was suspended in 50 ml of 0.2 M glycine-HCl buffer, pH 2.2 containing 10 mg of crystalline pepsin (Sigma), and incubated at 37 C for 60 min. Then it was washed successively with 1% SDS solution, 6 M urea solution and water. Washings with SDS at this stage was carried out because it was more effective than washing with water alone for removing enzyme protein and hydrolyzed products from the PG preparation. As shown in Table 4, the amount of the amino acid and amino sugar constituents of PG were in general relatively high compared with their amounts at Step 1 but the amounts of unrelated amino acids were still higher than those found so far in PG general from other gram negative organism. The recovery at this stage was 5.4% dry weight of the starting whole cells.

3) Step 3 (papain digestion by the method of Mandelstam, 1962). The preparation from Step 2 was suspended in 10 ml of 0.05 M phosphate buffer, pH 7.0 containing 0.01 M EDTA, 1 mg of cysteine-HCl and 10 mg of twice crystallized papain (Sigma) and incubated at 37 C for 1 hr. Then it was washed with SDS and urea as in Step 2. An electronmicrograph of this preparation (Fig. 9) showed rod shaped membranous structure with a smooth, glossy surface. The electron-dense, round bodies in the membranous structures seemed to be PHBA inclusions and will be described later. Chemical analysis showed

TABLE 4. *Amino acid and amino sugar compositions of preparations of murein sacculi of V. parahaemolyticus A55 at each purification step*

Constituents[a]	Preparation of murein sacculi at							
	Step 1		Step 2		Step 3		Step 5	
	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio
A_2pm	116	1.0	209	1.0	257	1.0	491	1.0
Alanine	710	6.1	564	2.7	588	2.3	818	1.7
Glutamic acid	611	5.3	328	1.6	223	0.9	431	0.9
Muramic acid	65	1.3	148	0.7	175	0.7	288	0.6
Glucosamine	134	1.8	207	1.0	204	0.8	213	0.4
Aspartic acid	417	3.6	147	0.7	66	0.3	73	0.2
Lysine	153	1.3	94	0.5	39	0.2	56	0.1
Arginine	119	1.0	45	0.2	23	0.1	15	0[b]
Threonine	215	1.9	62	0.3	30	0.1	25	0.1
Serine	224	1.9	95	0.5	67	0.3	46	0.1
Glycine	344	3.3	196	0.9	89	0.4	102	0.2
Valine	197	1.7	30	0.1	27	0.1	16	0[b]
Isoleucine	162	1.4	30	0.1	19	0.1	14	0[b]
Leucine	336	2.9	100	0.5	39	0.2	29	0.1
Tyrosine	135	1.2	90	0.4	23	0.1	15	0[b]

[a] Estimated with an amino acid autoanalizer. Values were not corrected for loss during hydrolysis.
[b] <0.05.

that the amount of most of the amino acid and amino sugar constituents of PG were higher relative to the amount of other amino acids than at Step 1 or 2 (Table 5). The molar ratio of the main constituent amino acid and amino sugar in PG was very close to the value expected for gram negative bacterial PG in general. The recovery at this stage was 4.7% by dry weight of the starting material. As shown in Table 3, the relative amounts of hexose and PHBA increased during purification from Step 1 to 3. In the Step 3 preparation, the PG component accounted for only one third of the total weight, the rest being sugar and PHBA. PHBA is apparently an energy reserve in the cytoplasm, but it is uncertain whether the sugar is a structural component of the cell walls or not at this stage.

4) Step 4 (removal of PHBA inclusions). The electron dense inclusions shown in Fig. 9 disappeared on extraction with chloroform and PHBA was identified chemically in the chloroform extract (Tamura et al., 1968). One gram of the preparation from Step 3 was extracted with 40 ml of chloroform at 65 C for 16 hr in a Soxhlet apparatus. PHBA was recovered in the extract and the residue was washed successively with acetone and water. Care was required to prevent the material from drying after chloroform treatment, because if it did so it aggregated into a hard lump which could not be made into a homo-

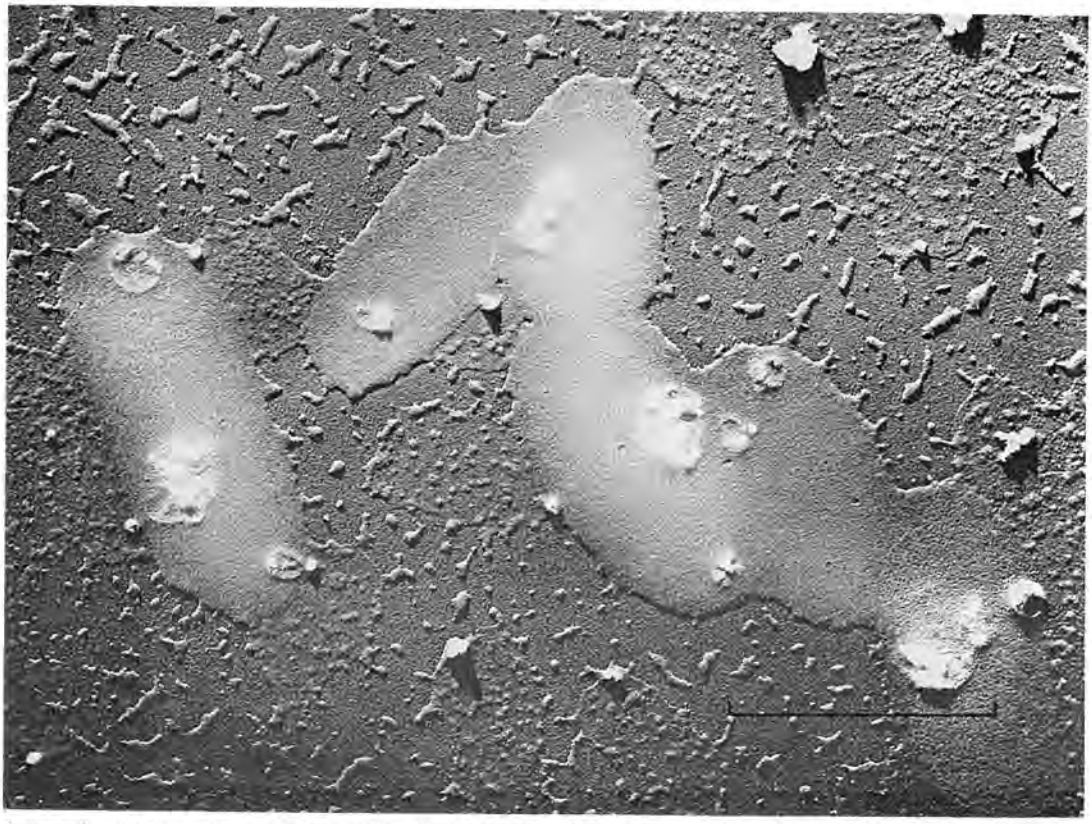

FIGURE 9. Shadowed preparation of a Step 3 preparation of *V. parahaemolyticus* A55. This preparation still contains polyglucose and PHBA. Rod shaped membranous sacculi with electron dense granules can be seen. The particles scattered in the background are probably murein degraded during the preparation procedure. Scale: 1 μm.

geneous suspension.

5) Step 5 (amylase treatment). Weidel and Pelzer (1964) reported that *E. coli* murein sacculi contained about 20% by dry weight of glycogen and that this could be removed by digestion with α-amylase. The polyglucose found in the present work differed from glycogen in many respects. For instance, it was not susceptible to either α-amylase or β-amylase. However, it could be digested by iso-amylase or pullulanse (Tamura et al., 1969). Therefore, the preparation from Step 4 (100 mg) was subjected to simulataneous digestion with 5,000 units of *Pseudomonas* isoamylase (Harada et al., 1968) and 2 mg of crystalline β-amylase (Sigma) in 50 ml of 0.01 M acetate buffer, pH 4.0 at 37 C for 16 hr. After the reaction, the mixture was washed with SDS and urea as described above. The resulting pellet had a transparent, jelly-like appearence. The yield at this stage (final preparation) was 1.1% by dry weight of the starting intact whole cells. The amino acid and amino sugar composition of the preparation at Step 5 (Table 4) was essentially the same as that at Step 3 except that the relative amounts of PHBA and polyglucose were much less. The main amino acids and amino sugars of the murein sacculus were glucosamine, muramic acid, alanine, glutamic acid, A_2pm in a molar ratio of 0.4: 0.6: 1.7: 0.9: 1.0. These values were not corrected for destruction during acid hydrolysis. The very low recovery of the final preparation, shown in Table 4, might due to aggregation of material during prolonged extraction with chloroform at high temperature. The material was too

TABLE 5. *Amino acid and amino sugar compositions of the peptidoglycans or murein sacculi obtained from various gram negative organisms by direct SDS digestion of whole cells*

Constituents	*V. parahaem.* A55		*E. coil* B[a]		*Pseudomonas* sp. 101[a]		*Spirillum serpens*[b]		Marine *Pseudomonas* sp. B-16[b]	
	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio	nmoles/mg	Molar ratio
A_2pm	492	1.0	932	1.0	498	1.0	470	1.0	820	1.0
Alanine	914	1.9	1,218	1.3	652	1.3	910	1.9	1,840	2.2
Glutamic acid	427	0.9	700	0.8	455	0.9	480	1.0	1,040	1.3
Muramic acid	234	0.5	504	0.5	422	0.9	320	0.7	1,290	1.6
Glucosamine	387	0.8	626	0.7	488	1.0	470	1.0	1,230	1.5
Aspartic acid	90	0.2	42	0.1	18	0[d]	100	0.2	tr	—
Lysine	85	0.2	103	0.1	24	0.1	39	0.1	—	—
Arginine	45	0.1	16	0[d]	tr[e]	—	47	0.1	—	—
Threonine	28	0.1	34	0[d]	7	0[d]	1	0[d]	20	0[d]
Serine	50	0.1	14	0[d]	tr	—	37	0.1	20	0[d]
Glycine	132	0.3	74	0.1	167	0.3	47	0.1	320	0.4
Valine	38	0.1	34	0[d]	tr	—	38	0.1	—	—
Isoleucine	19	0	6	0[d]	tr	—	34	0.1	—	—
Leucine	47	0.1	14	0[d]	tr	—	35	0.1	tr	—
Tyrosine	41	0.1	tr	—	tr	—	33	0.1	—	—

[a] Obtained in the same manner as for *V. parahaemolyticus* A55, except that Step 4 and 5 were omitted.
[b] Kollenbrander et al. (1968).
[c] Forsberg et al. (1972).
[d] >0.05.
[e] Trace, <0.01.

tough to be hydrolyzed completely by acid hydrolysis and so was ashed. An electronmicrograph of the preparation at Step 5 (Fig. 10) showed very thin, glossy membranous structures which still retained a rod-shape. These structures appeared to correspond to those designated by Weidel et al. (1964) as ' murein sacculi ' in *E. coli* and other gram negative enteric bacilli.

FIGURE 10. Shadowed preparation of Step 5 preparation of *V. parahaemolyticus* A55 free from polyglucose and PHBA. The rod shape can be seen but its outline is irregular. Scale: 1 μm.

The polyglucose may be storage material in the cytoplasm, like glycogen of *E. coli*, or may be an analogue of it. However, the chemical characteristics of the polyglucose were significantly different from those of *E. coli* glycogen, so the polyglucose may be a structural component of the cell walls of *V. parahaemolyticus* or closely associated with them. If so, it may actually be linked to PG.

6. *Separation of polyglucose from PG by gel filtration*

The preparation from Step 3 (100 mg) was digested with L-11 enzyme as described in the Materials and Methods. This enzyme splits muramyl-L-alanine linkages and so should split PG into the peptide moiety and glycan. The digested material was subjected to chromatography on Sephadex G-50 column. As shown in Fig. 11, about two thirds of the peptides in terms of total amino groups were eluted in a second peak of material (P-2) with a small amount of hexose but no amino sugar. The first peak of material (P-1), contained most of the hexose and amino sugar with some of the amino groups. The fractions in P-1 were combined and again applied to a Sephadex G-100 column. All the amino groups and amino sugars and the hexose were eluted together soon after the void volume (results not shown). This does not necessarily indicate that polyglucose is connected to PG because the glycan portion may be large enough to

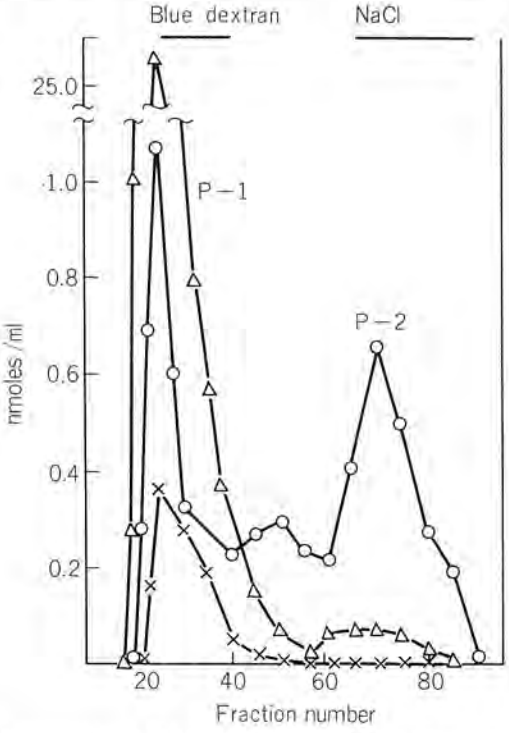

FIGURE 11. Gel filtration profile on Sephadex G-50 of Step 3 preparation of *V. parahaemolyticus* A55 solubilized with the L-11 enzyme. 100 mg of Step 3 preparation were hydrolyzed with the L-11 enzyme as described in the text. The insoluble residue was removed by centrifugation at 11,500$\times g$ for 30 min and the supernatnat was applied to a 100$\times$1.5 cm of Sephadex G-50 column and the column was eluted with water. Fractions of 2 ml were collected and assayed for total amino groups (○——○), hexosa mine (×——×) and hexose (△——△).

break through the column. The very large amout of hexose in the fractions made colorimetric assays impossible so this fractions was not studied further.

The PG preparation from Step 5 which had been freed from polyglucose was digested with the *Charalopsis* enzyme and analyzed by Sephadex gel filtration. As shown in Table 3, the Step 5 preparation still retained a small amount of hexose, even after extensive digestion with iso- and β-amylase. This hexose may have some structural significance because

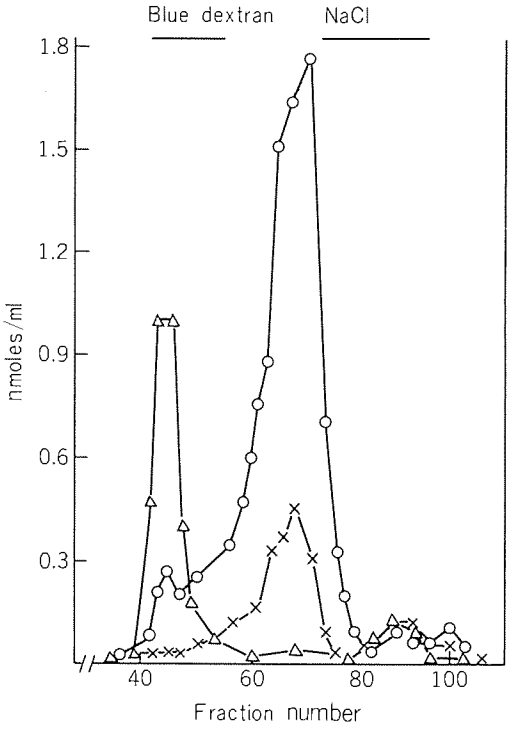

FIGURE 12. Gel filtration profile on Sephadex G-50 G-50 and G-25, connected in series of Step 5 preparation of *V. parahaemolyticus* A55 solubilized with *Charlopsis* enzyme. 100 mg of Step 5 preparation were digested with *Charalopsis* enzyme as described in the text and applied to 100 × 1.5 cm columns of Sephadex G-50, G-50 and G-25 columns connected in series. The columns were eluted with water. Fractions of 7 ml each were collected and assayed for total amino groups (○——○), hexosamine (×——×) and hexose (△——△).

the molar ratio of hexose to A_2pm approximately 1:1. If polyglucose is linked chemically to PG, this hexose which is resistant to amylase may be the terminal unit of polyglucose connecting the latter to PG. The preparation from Step 5 (100 mg) was digested with *Charalopsis* enzyme as described in the Materials and Methods and applied to Sephadex G-50, G-50 and G-25 columns connected in series. The *Charalopsis* enzyme hydrolyses the linkage of *N*-acetylmuramyl-β-1,4-*N*-acetylglucosamine in the glycan portion of PG producing a disaccharide-peptide monomer or its complexes. As shown in Fig. 12, the hexose compound was separated by chromatography from disaccharide-peptide complex and a negligible amount of hexose was found in the latter. Some of the amino groups appeared to be eluted in earlier fractions with the sugar portion, but this was due to a false reaction by the yellow color of hydrolyzed sugar because no amino groups were detected in these fractions by thin layer chromatography. The sugar eluted in earlier fractions seemed to be another kind of polyglucose which was not susceptible to α- or β-amylase nor isoamylase. It was probably a new kind of limit dextrin for isoamylase and not a cell wall component. However, it has not been studied further. No phosphorus was found in any of the fractions from the Sephadex columns, indicating the absence of linkages involving phosphorus. This finding seems significant because components of the cell wall other than PG are linked through phosphodiester bonds to the muramic acid residue in PG (Liu and Gotschlick, 1967).

7. *Action of the PG hydrolytic enzyme on the PG of V. parahaemolyticus*

Unless otherwise specified we used the Step 3 preparation containing PHBA and polyglucose in subsequent studies, because these non-PG components do not seem to interfere with the action of the hydrolytic enzyme and the procedures used to remove them may damage PG, partly because aggregates were formed

during chloroform extraction and partly because of the possibility of contamination by hydrolytic enzymes in the amylase preparation. For instance, we have found endo-*N*-acetylglucosaminidase as well as muramidase in crude barely β-amylase (Iwata et al., 1972) and it may also be present in purified amylase. As shown in Fig. 13, the PG preparation liberated NH_2 terminal amino groups or reducing groups with concomitant decrease in turbirdity on treatment with these enzymes. Lysozyme released 0.16 moles of reducing groups, the L-11 enzyme released 0.66 moles NH_2 terminal alanine and the L-3 enzyme released 0.26 moles NH_2 terminal alanine and 0.2 moles -NH_2-A_2pm. The ALE enzyme released 0.3 moles NH_2 terminal alanine. The values for terminal groups liberated by these enzyme are expressed as molar ratios to the total glutamic acid residues in the PG preparation. Intact PG had no detectable amount of free NH_2 terminal alanine, but 0.41 moles of A_2pm residue was found in it. Thus about half the peptide subunits in PG of *V. parahaemolyticus* are cross-linked.

All these findings indicate that the PG of *V. parahaemolyticus* A55 is a A_2pm type PG, like those of the cell walls of gram negative enteric bacteria such as *E. coli*. It also resembles PG of *E. coli* in chemical structure as will be reported in detail (Kato et al. 1976).

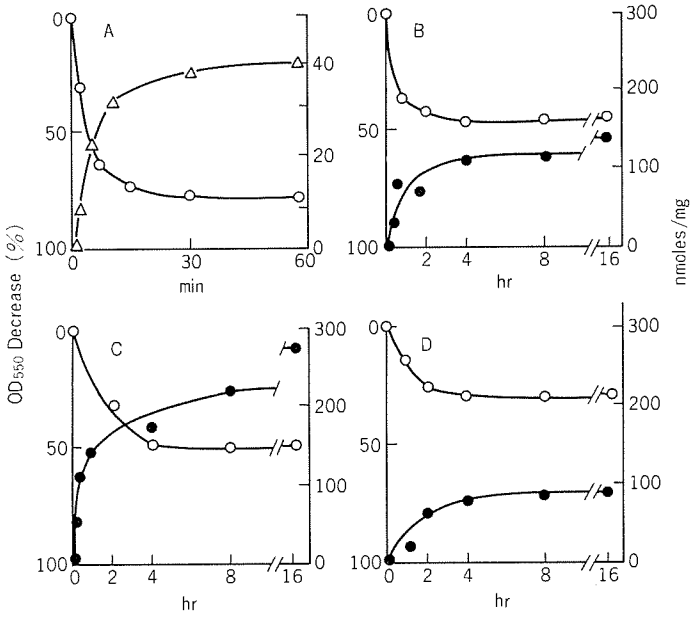

FIGURE 13A, 13B, 13C and 13D. Kinetics of hydrolyses of the Step 3 preparation of *V. parahaemolyticus* A55 by various enzymes. The experimental conditions were as described in the Materials and Methods. A: Lysozyme. B: L-11 enzyme. C: L-3 enzymes D: ALE enzyme. OD_{550} (○——○), free amino groups (●——●), reducing groups (△——△).

DISCUSSION

Some halophilic bacteria are said to be distinguished from most terrestrial organisms by the nature of the PG layer of their envelope. One extreme example is a *Halobacterium* species which is completely devoid of PG (Kushner, 1964). A marine pseudomonad B-16 was found to contain less PG than ordinary terrestrial gram negative organisms (Forsberg et al., 1972). Buckmire and MacLeod (1965) found that a quarter of the PG component in the cell envelope of a marine pseudomonad was released non-enzymatically when the organism was suspended in solution of low salt concentration and they suggested that the PG layer of this organism was made up units, which only form a continuous layer when their negative charges are neutralized by cations of a salt. Another example of an incomplete murein sacculus was reported by White et al. (1968). They found that whole vegetative cells of *Myxococcus xanthus* contained only 0.86% of PG by weight and concluded that this was not enough to form an intact murein sacculus. *V. parahaemolyticus* A55 is distinguishable from marine pseudomonads by the fact that its envelope liberates only about 2% of the total A_2pm in medium defi-

cient in salts, although its general structure may be damaged under these conditions. The fragmented appearence of PG obtained from either envelopes-I (prepared in the presence of protecting NaCl) or envelopes-II (prepared by cell lysis in solution of low salt concentration) can not be regarded as conclusive evidence that the PG layer is discontinuous since it was apparently an artifact, because using a reviced method intact murein sacculi were obtained which appeared identical to those of Enterobacteriaceae described by Weidel and Pelzer (1964). Further evidence for closed bags of PG in the organism was that PHBA inclusions remained in the PG preparation even after repeated washing with high and low speed centrifugation (Table 4), whereas a PG preparation derived from the cell envelopes was entirely devoid of PHBA (Table 2).

The final yield of the PG was 1.1% by weight of the cells. This value would represent the total PG content of the organism if the PG was isolated quantitatively. If so, the percentage yield of PG from the organism was close to the PG content of marine pseudomonad B-16 (Forsberg et al., 1972) and that of *Vibrio fetus* (Winter et al., 1971), but about half as much as that of *E. coli* (White et al., 1968) or *Spirillum serpens* (Kolenbrander and Ensign, 1968). However, the PG content of the organism which is presently available should be interpreted with caution until direct method for estimating exact amount of PG in the whole cells will be established.

The basic constituent amino acids and amino sugars found in PG of *V. prahaemolyticus*, as well as mode of action of serveral known PG hydrolytic enzymes to it, suggest that this PG is of the same typical and uniform chemotype as the PG of all other gram negative bacteria examined so far. There have been few attempts to isolate PG or the murein sacculus from organisms of the genus *Vibrio*. Hisatsune et al. (1972) has described that the Inaba and Ogawa strains of *Vibrio cholerae* had *E. coli* type PG, and that one of their PG specimens did not reveal a bag-shape morphologically.

Some amino acids that are not main components of PG were found in purified PG of *V. parahaemolyticus* and other organisms (Table 5). Those were glycine, lysine and sometimes aspartic acid, which are found as minor constituents in cross bridges in the peptide subunits of some gram negative bacteria, but were presumably not involved in cross bridges of *V. parahaemolyticus*. In *E. coli* and *Proteus mirabilis*, a lipoprotein attached covalently to PG (Martin and Frank, 1962) is an important constituent of a rigid layer, but lipoprotein seems to be less important for this purpose among Pseudomonadales. PG of *Spirillum serpens* forms a naked murein sacculus devoid of any covalently attached protein because a single treatment with aqueous SDS removes all the protein layer and leaves pure PG. Winter et al., (1971) isolated PG of *Vibrio fetus* in high purity solely by treatment with SDS, and also suggested the absence of covalently bound protein in this organism. In the case of *V. parahaemolyticus*, PG could not be obtained by a single extraction with SDS. As can be seen in Table 5, the Step 1 preparation contained appreciable quantities of amino acids which were not constituents of PG but which could only be removed by successive treatments with proteases. One possible explanation for the large amount of these amino acids is that considerable amounts of cytoplasmic materials were still trapped in the closed bag of the murein sacculus and were not digested by SDS, indicating a disadvantage of this method. However, it seems likely that protein may be attached covalently to PG in this organism. Studies on this special protein are now in progress.

The hexose moiety was separated from PG by gel filtration, so the polyglucose may be cytoplasmic material distinct from the PG. Polyglucose can also be separated from PG by ion exchange column chromatography (unpublished observation).

During lysis of some halophilic bacteria in hypotonic media deformation of the cell oc-

curs first and this leads to fragmentation of the cell (Kushner, 1964). However, during lysis of *V. parahaemolyticus* A55, cytoplasmic material leaked out but the shape of the cell remained unchanged. This suggests that the general barrier function of the cell envelope is damaged rather than that the cell is ruptured by sudden change of osmolarity. Scherrer and Gerhardt (1973) have demonstrated that the porosity of the cytoplasmic membrane of *Bacillus megaterium* was controled by Mg^{++}. It seems possible that deprivation NaCl may cause a increase in the porosity of the envelope of *V. parahaemolyticus* without affecting PG layer. Buckmire and MacLeod (1965) suggested that the membrane of halophiles consists of polyanionic molecules which can only be stabilized by the neutralizing effect of cations of salt. Thus when electron negative group of the molecule is exposed by deficiency of neutralizing cations, the resulting repulsive forces between these anionic molecules result in widening the porosity in the membrane. The stabilizing effect of low pH can be explained by supposing that dissociation of the anionic residues of the molecule is reduced at low pH. This does not mean that acidic conditions are optimal for *V. parahaemolyticus*, for in fact, the organism requires a weak alkaline environment for optimal growth and acidity is extremely adverse for growth and survival. The PG of *V. parahaemolyticus* is apparently not affected by salt since the intact murein sacculus was obtained even though the preparation was washed repeatedly with distilled water, except when the cells were suspended in SDS solution for the first time. Moreover the PG was not affected by further treatment with 100 mM EDTA solution (unpublished observation). Therefore, it is most improbable that the PG layer in the envelope of the organism is directly responsible for lysis of the cells in solution of low salt concentration.

An electronmicrograph of a sectioned preparation of lysed cell showed that the inner and outer layers were considerabley separated from each other. Buckmire and MacLeod (1965) suggested that in the marine pseudomonad they examined some material effectively held PG and the other layers together in solution of high salt concentration but that it disappeared in medium of lower salt concentration. This might also be the case in *V. parahaemolyticus*. In electronmicrograph we could not see any layer corresponding to PG in a sections of intact cells of the organism prepared by the method of Murray et al. (1965). This agrees with the report of Koga et al. (1976). The sections of several marine vibrio including *V. parahaemolyticus* made by Murray's method also showed no structure corresponding to PG (Colwell, 1970). Forsberg et al. (1972) suggested that the difficulty in detecting a densely stained PG layer in the marine pseudomonad B-16 may be partly due to its thinner nature than that of *E. coli* and partly due to the closer adherence of PG to the outside surface of the cytoplasmic membrane than to the outer layer. If the PG of *V. parahaemolyticus* is similar to those of marine organisms, it must differ from the PG of enteric bacteria, since electronmicrography shows that the latter associated with the outer layer.

The method employed to isolate PG from

FIGURE 14. Shadowed preparation of a murein sacculus of *Pseudomonas* 101. This preparation corresponds to Step 2 preparation of *V. parahaemolyticus* A55. The membranous structure is very thin with a faint outline. Scale: 1 μm.

intact cells of *V. parahaemolyticus* was tested on *E. coli* B (our laboratory strain) and *Pseudomonas* sp. 101 to examine its general availability. The latter strain was supplied by courtesy of Prof. M. Masui, Osaka City University. It requires 10% NaCl to maintain its normal physiological state (Masui et al., 1973) and therefore can be classified as a moderate halophile. Figure 14 shows the murein sacculus obtained from strain 101 by direct SDS digestion of whole cells without mechanical disintegration. A pure murein sacculus was also obtained from *E. coli* B by this method. Analytical data on the composition of these preparation are shown in Table 5. Data of other PG preparations made by other authors are also listed in Table 5 for comparison. The composition of all the PG samples listed in the table seemed to be identical except for their contents of some minor amino acids.

Further chemical analysis of the nature of the cross linkage in the peptide and the chain length of the glycan of the PG of *V. parahaemolyticus* A55 will be reported (Kato et al. 1976).

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