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リゾチーム処理 *Micrococcus lysodeikticus* 細胞壁非透析分画の研究*

友田正司

Studies on the non-dialyzable fraction obtained from cell walls of *Micrococcus lysodeikticus* by treatment with lysozyme.

Masashi Tomoda

(Summary)

The non-dialyzable fraction obtained from cell walls of *Micrococcus lysodeikticus* by treatment with lysozyme has been purified into three fractions by application of a fractional precipitation method using cetyl pyridinium chloride solution. Each fraction contains D-glucose, N-acetyl D-muramic acid, N-acetyl D-glucosamine, N-acetyl D-muramic acid 6-phosphate and N-acetyl D-glucosamine 6-phosphate as the component carbohydrate, and has the pentapeptide having molar proportions of alanine 2, glutamic acid 1, glycine 1 and lysine 1. The significant differences on the chemical properties of fractions were found in the amounts of glucose, N-acetylglucosamine 6-phosphate and the peptide. From the results of the treatments with two phosphatases, a phosphodiesterase and trichloroacetic acid, it is conceivable that the both amino sugar phosphates form phosphodiester linkages between the peptidoglycan, consisted of alternating N-acetylglucosamine and N-acetylmuramyl peptide residue, and the other glucose-rich polymer.

Introduction

The cell wall constituents of *Micrococcus lysodeikticus* are the famous traditional substrate of lysozyme. Although the substrate of lysozyme is known to be distributed throughout both Gram-positive and Gram-negative groups of bacteria, *Micrococcus lysodeikticus* is the most sensitive species.[1] Cell walls of *Micrococcus lysodeikticus* are almost completely degraded to soluble products on digestion with lysozyme and the complex mixture of fragments is able to be separated on the basis of diffusability through dialysis tubing into dialyzable and non-dialyzable fractions.

Several important informations on the structure of the cell wall have come from investigations of the dialyzable fraction. A disaccharide was obtained and the structure was proposed independently by Salton and Ghuysen,[2,3] Perkins[4] and Hoshino,[5] but they did not

* この研究は Laboratory for Carbohydrate Research, Harvard Medical School and Massachusetts General Hospital, U.S.A. において 1967 年 8 月から約 1 年間行なった.

1) M.R.J. Salton and J.G. Pavlik : Biochim. Biophys. Acta, **39**, 398 (1960).
2) M.R.J. Slaton and J.-M. Ghuysen : Biochim. Biophys. Acta, **36**, 552 (1959).
3) M.R.J. Salton and J.-M. Ghuysen : Biochim. Biophys. Acta, **45**, 355 (1960).
4) H.R. Perkins : Biochem. J., **74**, 182 (1960).
5) O. Hoshino : Chem. Pharm. Bull., **8**, 405, 411 (1960).

arrive at the correct conclusion. Subsequently, the disaccharide was isolated on a large scale by Sharon, Osawa, Flowers and Jeanloz,[6] and it was concluded that 4–β–N–acetylglucosaminyl N–acetylmuramic acid is the most suitable one for the structure. A tetrasaccharide was also obtained by Salton and Ghuysen[2] from the dialyzable fraction of *Micrococcus lysodeikticus* cell walls. Now the structure of this compound is recognized to be a dimer of the disaccharide. More recently, the isolation of new type oligosaccharides was reported by Hoshino and Jeanloz.[7] In addition to the disaccharide and tetrasaccharide, several peptidoglycans were isolated from the dialyzable fraction. Ghuysen[8] studied two of peptidoglycans having identical molar proportions of disaccharide 1, alanine 2, glutamic acid 1, glycine 1 and lysine 1. The disaccharide–pentapeptides were recently isolated and the structures of them were proposed by Mirelman and Sharon.[9,10] The presence of the sequence of 4–β–N–acetylglucosaminyl N–acetylmuramic acid –L–alanine –D–glutamic acid –L–lysine –D–alanine in peptidoglycans from cell walls of many species of bacteria have been reported. In addition to *Micrococcus lysodeikticus*, those contain *Staphylococcus aureus*,[11,12] *Lactobacillus casei*,[13] *Streptomyces albus*,[14] *Micrococcus reseus*,[14] *Pneumococci strain R36A*,[15] *Arthrobacter crystallopoietes*,[16,17] *Streptococcus faecalis*,[18] *Streptococcus pyogenes*,[19] *Micrococcus flavis*,[20] *Micrococcus citreus*,[20] and *Sarcina lutea*.[20], Like the peptidoglycans from *Micrococcus lysodeikticus* walls, some of them possess glycine as the component of peptide and some possess D–glutamine instead of D–glutamic acid. A new pentapeptide dimer was isolated from *Micrococcus lysodeikticus* by means of an enzymatic hydrolysis.[21] Thus, the chain composed of both peptide–substituted and unsubstituted N–acetylmuramic acid residues linked to N–acetylglucosamine is considered to be the backbone structure of the cell walls of many bacteria.

Studies on the dialyzable fraction of a lysozyme digest of *Micrococcus lysodeikticus* walls have given us many valuable informations on the backbone structure of the cell wall.

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21) J.–M. Ghuysen, E. Bricas, M. Lache and M. Leyh–Bouille : Biochemistry, **7**, 1450 (1968).

But the yield of dialyzable fraction is less than one third of the original cell walls and that of non-dialyzable fraction is about two third of the material. Many unsolved problems are still remained within the non-dialyzable fraction.

In this report, the properties and the role of amino sugar phosphates in the non-dialyzable fraction will be described. The material was purified into three fractions, then amino sugar components of the fractions were determined using Dowex 50 column chromatography after hydrolysis. The manner of phosphodiester linkages in the main fraction was investigated by treatment with trichloroacetic acid, followed by fractionations using an ion exchange chromatography and a gel filtration.

Experimental Procedure

Non-dialyzable Fraction——Commercially available cells of Micrococcus lysodeikticus (Miles Chemical Co., Elkhart, Indiana, U.S.A.) were used as the starting material. They were disintegrated in the Sorvall Omni-Mixer high speed homogenizer, and the walls were isolated according to the method of Sharon and Jeanloz.[22] The preparation of non-dialyzable fraction of lysozyme digest of the cell walls was carried out in the same way as that described by Sharon, Osawa, Flowers and Jeanloz.[6]

Thin-layer Electrophoresis——10 g of Kieselguhr G (E. Merck Co., Darmstadt, Germany) were mixed with 20 ml of a buffer solution and stirred well. Glass plates (5 × 20 cm) were coated with 0.25 mm thick layer by the use of an applicator. Each thin-layer plate was used after 30 min. standing at room temperature. Electrophoresis was carried out with pyridine-acetic acid buffer of pH 4.3 (pyridine : acetic acid : water, 1 : 2 : 97) at the condition of 10 volt per cm for 2 hr. and with 0.1M borate buffer of pH 9.0 at the same voltage for 3 hr. Samples were applied to the center of each plate. Anisaldehyde sulfuric acid reagent[23] was used for detection.

Thin-layer Chromatography——20 g of Avicel Micro Crystalline Cellulose (Brinkmann Instruments Inc., Westbury, New York, U.S.A.) were mixed well with 90 ml of water in a homogenizer and glass plates (20 × 20 cm or 10 × 20 cm) were coated with 0.25 mm thick layer by the use of an applicator. The plates were stored overnight at room temperature before use. Samples were spotted in line at 1.5 cm from an edge and the solvents were allowed to ascend to a height of 10 cm from starting point at 22°. Ninhydrin, silver nitrate,[24] *p*-anisidine[25] and Ehrlich[26] reagents were used for general detection. Hanes-Isherwood reagent[27] was used for the detection of phosphate esters.

Gas Chromatography——0.1 ml of Tri-Sil reagent (Pierce Chemical Co., Rockford, Illinois, U.S.A.) was added to 50 μg of the dried sample and the resuting solution was kept at room temperature for 15 min., then redried. The residue was dissolved in 50 μl of heptane and 1 μl of the soltion was applied to gas chromatography using Perkin-Elmer model 900 with 3% OV17 on Chromosorb Q at 160°. 10 feet long copper tube was used.

Colorimetric Determination of Components——Ninhydrin reaction of free amino groups was carried

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out according to the method of Yemm and Cocking.[28] Hexosamine was determined by the modification of the Elson–Morgan reaction described by Rondle and Morgan[29]. Coloration of N–acetylamino sugars was determined by the modification of the Morgan–Elson reaction described by Reissig, Strominger and Leloir[30] with an additional modification. As the results of applying various heating times, the coloration was still increasing after 40 min. of heating, but the degree of increase was small after 30 min. in the case of this report, the heating time of 30 min. in a boiling water bah was therefore selected in the Morgan–Elson reaction. Glucose was determined by the method of Scott and Melvin,[31] and by the method of Dubois, Gilles, Hamilton, Rebers and Smith[32] for the measurement of eluates of column chromatographies. Determination of reducing sugars was carried out according to the method of Park and Johnson.[33] Phosphorus was determined by the method of Chen, Toribara and Warner.[34] The determination of amino acids was performed by Dr. G.L. Mechanic using an amino acid analyzer by the method of Piez and Morris[35] after hydrolysis with 6*N* hydrochloric acid at 110° for 20 hours.

Measurement of Radioactivity——This was carried out by Mr. S. Pancake using Deluxe Radiochromatogram Scanner Model RSC–363 (Baird–Atomic Inc., Cambridge, Massachusetts, U.S.A.)

Results

Preparation of Non-dialyzable Material——The preparation was performed by Dr. O. Hoshino and Miss M. Rascher. 10 to 20 g of lyophilized cell walls were digested with crystalline egg white lysozyme (1 to 2 mg of enzyme per g of walls in 30 to 40 ml of distilled water). The digestion was carried out under toluene for 4 hr. at 37°. The resulting solution was transfered into a cellophane bag and dialyzed against 2 volumes of distilled water at 37° for 24 hours. The dialysis was repeated several more times against similar volumes of fresh water. The solution retained in the dialysis bag was centrifuged at 9000 r.p.m. for 15 min. to remove a small amount of suspended impurities, and was lyophilized. From 10 g of cell walls, 6.0 to 6.5 g of non–dialyzable material were obtained.

Fractionation of Non-dialyzable Material——1.8 g of the material was dissolved in 5 ml of water, and 80 ml of 1% cetyl pyridinium chloride solution were added under stirring. A precipitate was obtained by centrifuging at 18000 r.p.m. for 30 min., then washed with 20 ml of 1% cetyl pyridinium chloride solution and centrifuged similarly. The washing was repeated once more. The precipitate was dispersed in 6 ml of 2*N* acetic acid and stirred for 6 hr. at room temperature. Then, 5 volumes of ethanol were added and centrifuged at 15000 r.p.m. for 20 min. The precipitate was washed with ethanol and centrifuged similarly, then dried in vacuo in a desiccator. The dried substance was dissolved in 15 ml of water and centrifuged at 18000 r.p.m. for 30 min. The supernatant was

28) E.W. Yemm and E.C. Cocking : Biochem. J., **58**, xii (1954).
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35) K.A. Piez and L. Morris : Anal. Biochem., **1**, 187 (1960).

lyophilized. This will be termed as F–I.

The supernatant and the washings with 1% cetyl pyridinium chloride solution were combined and centrifuged at 18000 r.p.m. for 30 min. Then, the precipitate was washed with 20 ml of the mixture of ethanol and 1% cetyl pyridinium chloride solution (4:1) and centrifuged similarly. The precipitate was dissolved in 4 ml of 2*N* acetic acid and stored overnight in a refrigerator, and then, after adding of 5 volumes of ethanol, it was centrifuged at 15000 r.p.m. for 20 min. The precipitate was washed with ethanol and centrifuged similarly. After drying in vacuo in a desiccator, it was dissolved in small amount of water and lyophilized. This will be termed as F–II

The supernatant and washings described above were combined and concentrated at 30° under reduced pressure to 6 ml, followed by gradual addition of 5 volumes of ethanol. After centrifuging at 10000 r.p.m. for 20 min., the precipitate was washed with 24 ml of the mixture of ethanol and 1% cetyl pyridinium chloride solution (5:1) and centrifuged similarly. The precipitate was dissolved in 8 ml of 2*N* acetic acid and stored overnight in a refrigerator, and then, after adding of 5 volumes of ethanol, it was centrifuged at 15000 r.p.m. for 20 min. The precipitate was washed with ethanol and centrifuged similarly. After drying in vacuo in a desiccator, the precipitate was dissolved in small amount of water and lyophilized. This will be termed as F–III. These procedure are shown in Chart 1.

The yields and homogeneities of three fractions differ from material to material. The results are shown in Table I.

The homogeneity was examined by thin-layer electrophoresis. All fractions moved to anodic side. The resulting distances from the starting point were 20 mm in F–I, 2 mm in F–II and 18 mm in F–III using pyridine–acetic acid buffer, and 27 mm in F–I, 2 mm in F–II and 24 mm in F–III using borate buffer. The homogeneity was also confirmed by Sephadex G–100 gel filtration.

TABLE I. Yields and homogeneties of F–I, F–II and F–III.

Material	Fraction	Yield (%)	Homogeneity
4LD (by Dr. Hoshino)	F–I	27.1	homogeneous
	F–II	18.5	heterogeneous
	F–III	31.0	homogeneous
7LD (by Dr. Hoshino)	F–I	16.0	homogeneous
	F–II	6.4	heterogeneous
	F–III	54.6	homogeneous
8LD (by Dr. Hoshino)	F–I	31.4	homogeneous
	F–II	17.4	homogeneous
	F–III	41.5	homogeneous
ND (by Miss Rascher)	F–I	10.9	homogeneous
	F–II	1.3	homogeneous
	F–III	79.5	heterogeneous

Chart 1

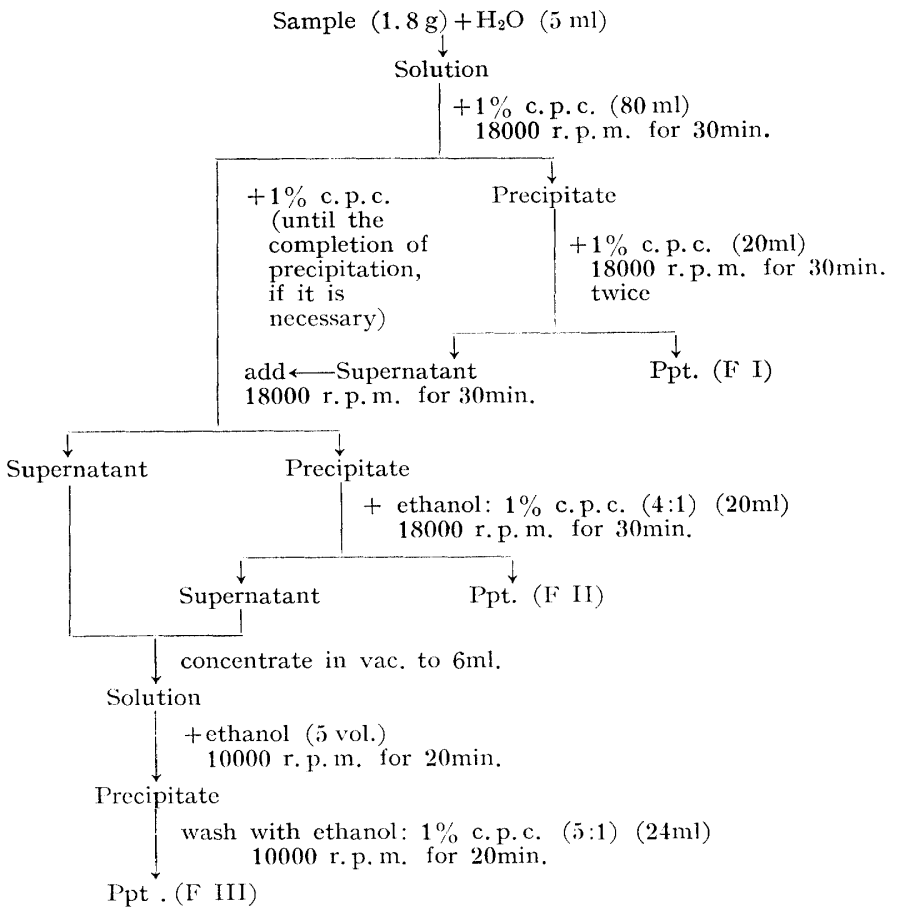

Chart 1. Fractionation of non-dialyzable material.

Isolation of Amino Sugar Phosphates from Acid Hydrolyzate——500 mg of the non-dialyzable material were hydrolyzed with 50 ml of 6*N* hydrochloric acid in a boiling water bath for 2 hours. After cooling to room temperature, the HCl was removed by rotary evaporation under reduced pressure and the residue was added with small amount of ethanol and evaporated similarly. This treatment with ethanol was repeated until the HCl was almost completely removed. Then, the residue was added with 10 ml of water and insoluble by-products were removed by centrifuging. After washing with 5 ml of water and centrifuging, the supernatants were combined and concentrated to 5 ml by rotary evaporation under reduced pressure. The solution was applied to a column (1.1×40 cm) of Dowex 50W-X8, H^+ form (200 to 400 mesh). The column was eluted with water at a flow rate of 1 ml per 3 min. Fractions of 2 ml were collected and analyzed by the method of Yemm and Cocking,[28] the method of Rondle and Morgan[29] and the method of Park and Johnson.[33] Aliquots of 0.1 ml were withdrawn for analysis by the ninhydrin reaction and of the reducing value, and 0.5 ml for analysis by the Elson-Morgan reaction. Elution curves are given in Fig. 1. The first peak will be termed as P-1, the second peak as P-2 and the third Elson-Morgan reaction positive peak as P-3. Fractions (P-1) comprising

17 to 32 effluent ml contained glucose and an unknown substance which shows the coloration having the maximum absorption wave length at 565 mμ by the Elson–Morgan reaction. Fractions (P–2) comprising 33 to 72 effluent ml contained muramic acid 6–phosphate, and fractions (P–3) comprising 101 to 142 effluent ml contained glucosamine 6–phosphate. They (P–2 and P–3) are separately concentrated and purified by applying again to the Dowex 50W–X8 column chromatography in the same way. The fractions contained the amino sugar phosphate were collected and concentrated under reduced pressure, then lyophilized. The yields were 0.8% in muramic acid 6–phosphate and 0.2% in glucosamine 6–phosphate.

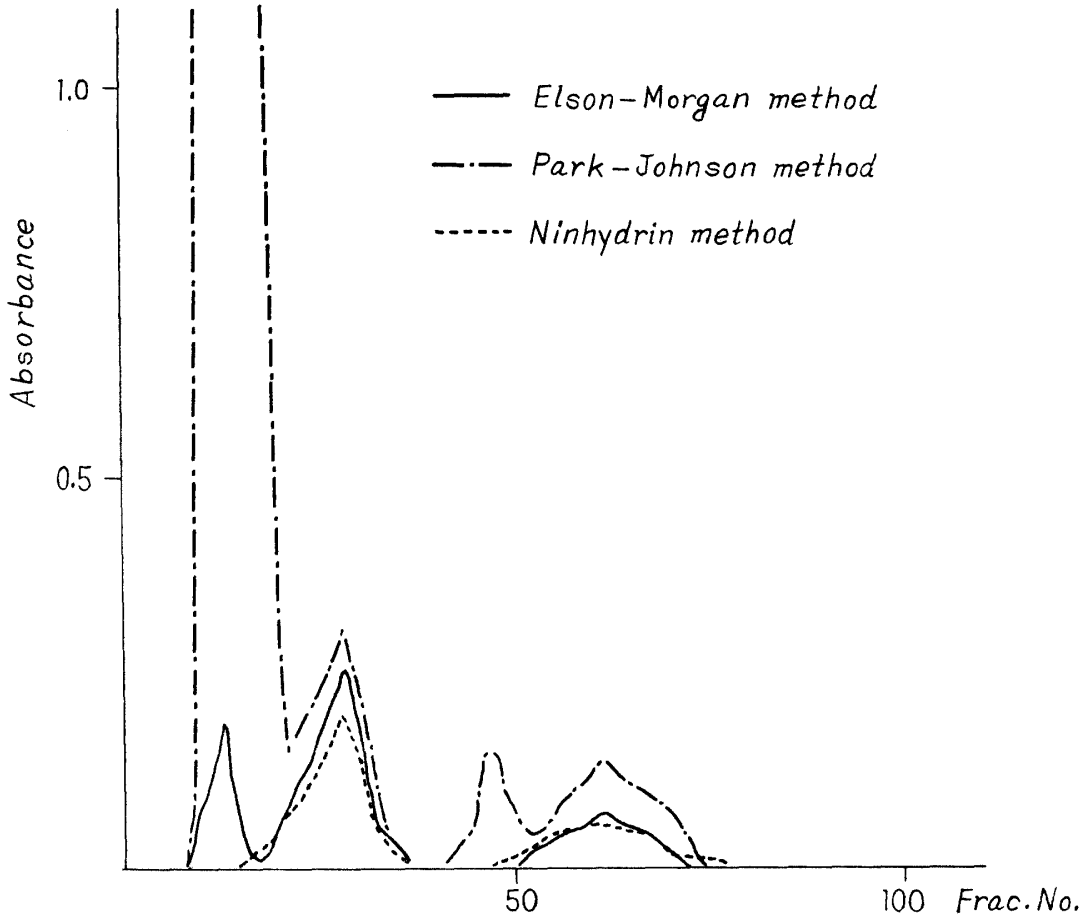

Fig. 1. Isolation of amino sugar phosphates by Dowex 50 chromatography.
In the case of the Elson–Morgan reaction, absorbances were measured at 565 mμ for P–1, at 510 mμ for P–2, and at 530 mμ for P–3.

Identification of Amino Sugar Phosphate——The purified fractions P–2 and P–3 gave respectively single spot on cellulose thin–layer chromatography using several solvent systems. They were detected by both ninhydrin and Hanes–Isherwood reagent.[27] The determinations of phosphorus[34] of the fractions P–2 and P–3 gave the results that the contents of phosphorus correspond respectively to that of muramic acid monophosphate

ester for P–2 and that of glucosamine monophosphate ester for P–3. The hydrolyzates of them with 6*N* hydrochloric acid in a boiling water bath for 4 hr. revealed muramic acid from P–2 and glucosamine from P–3 in addition to the original phosphate. Solvent systems of cellulose thin-layer chromatography and Rf values of them are given in Table II.

TABLE II. Rf values of amino sugars and their phosphates.

	Solvent A	Solvent B	Solvent C
glucosamine–HCl	0.55	0.27	0.56
galactosamine–HCl	0.50	0.25	0.61
mannosamine–HCl	0.58	0.26	0.60
muramic acid	0.61	0.48	0.45
glucosamine 6–phosphate	0.24	0.11	0.12
P–2 (muramic acid 6–phos.)	0.32	0.31	0.08
P–3	0.24	0.11	0.12

Solvent A, ethanol: 1*M* ammonium acetate, pH 3.8 (7:3).
Solvent B, n-butanol: acetic acid: water (2:1:1).
Solvent C, phenol: 1% ammonium hydroxide (2:1).

The Rf values of P–3 in several solvent systems correspond to those of authentic glucosamine 6–phosphate. Recently, however, a report that denies the presence of glucosamine 6–phosphate in the cell walls of Micrococcus lysodeikticus was published.[36] So further confirmations of amino sugar portion of fraction P–3 were carried out. 1 mg of the sample was dissolved in 5 ml of 6*N* hydrochloric acid and heated in a boiling water bath for 4 hours. After removal of the HCl by rotary evaporation under reduced pressure, the residue was dissolved in small amount of water and applied to a column (0.8×10 cm) of Dowex 50W–X8, H^+ form. The column was eluted with 40 ml of water, then eluted with 40 ml of 1*N* hydrochloric acid. The amino sugar phosphate was removed from water eluent. After removal of the HCl under reduced pressure from the eluent with 1*N* hydrochloric acid, the residue was used as the sample for the ninhydrin degradation method[37] and for gas chromatography.

Ninhydrin degradation method was carried out using cellulose thin-layer chromatography. The spots of the sample and standards applied to thin-layer plates were sprayed with a 2% solution of ninhydrin in 95% ethanol, containing 4% pyridine. The plates were then placed at 80° for 3 hr. in a "T.L.C. Chromatank" (Shandon Scientific Co. Ltd., Illinois, U.S.A.) containing a mixture of pyridine and water (1:1) in a beaker. Then, the plates were taken out, followed by usual development after removal of smell of pyridine. *p*–Anisidine reagent[25] was used for detection of the resulting pentoses. The results are given in Table III.

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37) P.J. Stoffyn and R.W. Jeanloz : Arch. Biochem. Biophys., **52**, 373 (1954).

TABLE. III. Rf values of the pentoses resulted from hexosamines.

	Solvent D	Solvent E
Glucosamine (or Mannosamine)⟶D-Arabinose	0.34	0.20
Galactosamine⟶D-Lyxose	0.43	0.29
P-3⟶	0.34	0.20

Solvent D, n-butanol: pyridine: acetic acid: water (60:40:3:30);
Solvent E, ethyl acetate: pyridine: water (10:3:2).

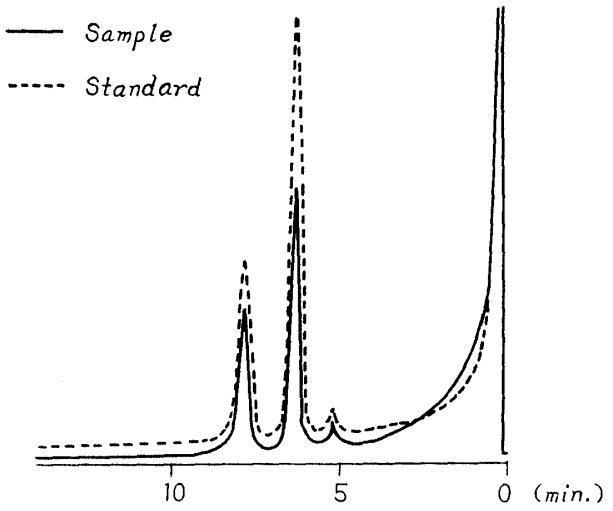

Fig. 2. Gas chromatogram of trimethylsilyl derivative of the hydrolyzate of P-3.

Finally a gas chromatographic technique was applied for the identification of the amino sugar. The gas chromatogram is given in Fig. 2. and is completely identical with that of trimethylsilyl derivative of D-glucosamine.

P-3 was also treated with a phosphatase. 220 μg of the sample were dissolved in 1 ml of 0.1M ethanolamine-hydrochloric acid buffer (pH 9.5) containing 0.05M magnesium acetate, and 10 μg of calf mucosa alkaline phosphatase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in 0.1 ml of the buffer were added. The solution was kept at 37° for 4 hr., then added with two drops of 1N hydrochloric acid to pH 5. 68.5% of the phosphate linkages in the sample were split. The solution was applied to a column (0.8×6 cm) of Dowex 50W-X8, H^+ form (200 to 400 mesh) and eluted with 20 ml of water, followed by 20 ml of 1N hydrochloric acid. The effluent with hydrochloric acid was used as the sample for the similar tests to the case of the acid hydrolyzate. The Rf values of ethanolamine hydrochloride which is contained in the sample are 0.37 in Solvent B and 0.80 in Solvent C. Therefore, it does not interfere the detection of amino sugar in the cases of using these solvent systems. In the case of gas chromatography, a peak which was derived from ethanolamine was appeared at very near position to the starting point. In these results, the amino sugar obtained from P-3 was shown to be completely identical

with authentic D-glucosamine.

Analysis of Components of Non-dialyzable Fractions——2 mg of samples were hydrolyzed with 0.3 ml of 4*N* hydrochloric acid at 100° for 1 hr. for analysis of the components. The acid was removed by rotary evaporation under reduced pressure as described above. In order to get clear results, the dried residue was dissolved in small amount of water and applied to a column (0.8×5 cm) of Dowex 50W-X8, H^+ form. The column was eluted with 20 ml of water, followed by 20 ml of 1*N* hydrochloric acid. Each effluent was concentrated under reduced pressure and examined by cellulose thin-layer chromatography. The HCl was removed in the same way. Neutral sugar and amino sugar phosphates were detected from the water effluent, and the other were revealed from the acid effluent. Rf values of components are shown in Table IV. The result was the same in the each fraction.

TABLE IV. Rf values of components of the hydrolyzate.

	Solvent B	Solvent C	Solvent F
glucose	0.32	0.28	0.43
glucosamine-HCl	0.27	0.56	0.31
muramic acid	0.48	0.45	0.36
glucosamine 6-phosphate	0.11	0.12	0.05
muramic acid 6-phosphate	0.31	0.08	0.07
alanine	0.45	0.54	0.19
glutamic acid	0.39	0.24	0.12
glycine	0.39	0.39	0.11
lysine	0.24	0.74	0.05
unknown*	0.65**	0.88**	0.67** 0.49**
	0.57**	0.27**	0.18*** 0.15**
	0.33**	0.19**	0.11*** 0.07***
	0.14***	0.09***	

Solvent F, ethyl acetate: pyridine: acetic acid: water (5:5:1:3).
* All of them were revealed from the acid effluent.
** Ninhydrin positive; *** Silver nitrate positive.

Determination of Components of Non-dialyzable Fractions——100 mg of the sample were hydrolyzed with 10 ml of 6*N* hydrochloric acid at 100° for 2 hr., then the HCl was removed by rotary evaporation. 2 ml of the solution of the hydrolyzate were applied to a column (1.1×40 cm) of Dowex 50W-X8, H^+ form (200 to 400 mesh). The column was eluted with 180 ml of water at a flow rate of 1 ml per 3 min. Fractions of 6 ml were collected and aliquots of 2 ml were withdrawn for analysis by Elson-Morgan reaction.[29] Absorbances at 510 mμ were measured for the determination of muramic acid 6-phosphate and those at 530 mμ were measured for the determination of glucosamine 6-phosphate.

After determinations of amino sugar phosphates, the coulmn was eluted with 180 ml

of 1*N* hydrochloric acid, then the HCl was removed from the effluent by rotary evaporation. The residue was dissolved in 2 ml of water and the solution was applied to a column (0.8 ×15 cm) of Dowex 50W-X8 buffered at pH 6.05 with sodium phosphate. The column had been prepared by washing the resin with each 15 ml of 1*N* sodium hydroxide, 5*N* hydrochloric acid, 1*M* sodium phosphate buffer (pH 6.05) and water until the effluent became phosphate free. The column was eluted with 30 ml of water followed by 50 ml of 1*N* hydrochloric acid. Fractions of 1 ml were collected and aliquots of 0.1 ml were withdrawn for analysis by Elson-Morgan reaction.[29] The water effluent contained muramic acid, and glucosamine hydrochloride was eluted with hydrochloric acid.

The sugar and amino acid compositions are given in Table V. Values of amino sugars and their phosphates are corrected for destruction during acid hydrolysis. 2.1% of hexosamine, 6.5% of the phosphate linkage of muramic acid 6-phosphate and 10.0% of the phosphate linkage of glucosamine 6-phosphate were decomposed respectively. Total N-acetyl hexosamine value was determined using the modified Morgan-Elson method[30] after treatment with 0.1*N* hydrochloric acid at 100° for 20 min. It is thinkable that the most of amino sugars are present as N-acetyl derivatives from these results.

Table V. Sugar and amino acid compositions (%) of non-dialyzable fractions.

	Non-dial. material	F-I	F-II	F-III
glucose	15.1	25.4	10.5	20.0
muramic acid	8.8	9.2	9.4	11.0
glucosamine	9.1	9.6	9.8	9.7
muramic acid 6-phosphate	0.88	0.97	1.25	1.35
glucosamine 6-phosphate	0.22	0.33	0.14	0.18
phosphorus (organic)	0.10	0.12	0.09	0.11
N-acetylhexosamine as GlcNAc	18.2			17.4
N-acetylhexosamine as MurNAc	29.8			28.4
alanine		1.1 (0.12*)	17.3 (1.94*)	9.0 (1.01*)
glutamic acid		0.7 (0.05*)	9.1 (0.62*)	7.2 (0.49*)
glycine		0.4 (0.05*)	6.9 (0.92*)	3.7 (0.49*)
lysine		0.9 (0.06*)	12.6 (0.86*)	6.7 (0.46*)

(*m Mol/g)

Treatment with Trichloroacetic Acid——In order to split a part of phosphate linkages and to separate resulting fractions, the treatment with trichloroacetic acid was attempted. The non-dialyzable material was dissolved in 100 times of 10% trichloroacetic acid and the solution was kept at 22°. Aliquots of the sample were withdrawn and diluted to ten times of volumn with water, then extracted thrice with equal volume of ether to remove

trichloroacetic acid. Upper layers were withdrawn and discarded, then aliquots of water layer were withdrawn for the measurement of reducing values by Park–Johnson method[33] and of N-acetyl hexosamine values by the modified Morgan–Elson reaction.[30] The increase of coloration by Morgan–Elson reaction ran almost parallel with that of reducing value. The phosphate linkages of amino sugar 6-phosphates were completely stable in a trichloroacetic acid solution. On the other hand, glucose 1-phosphate linkage was completely split by the treatment with trichloroacetic acid. These results are shown in Fig. 3. N-Acetyl hexosamine 1-phosphate is hydrolyzed with acids much more rapidly than glucose 1-phosphate.[38]

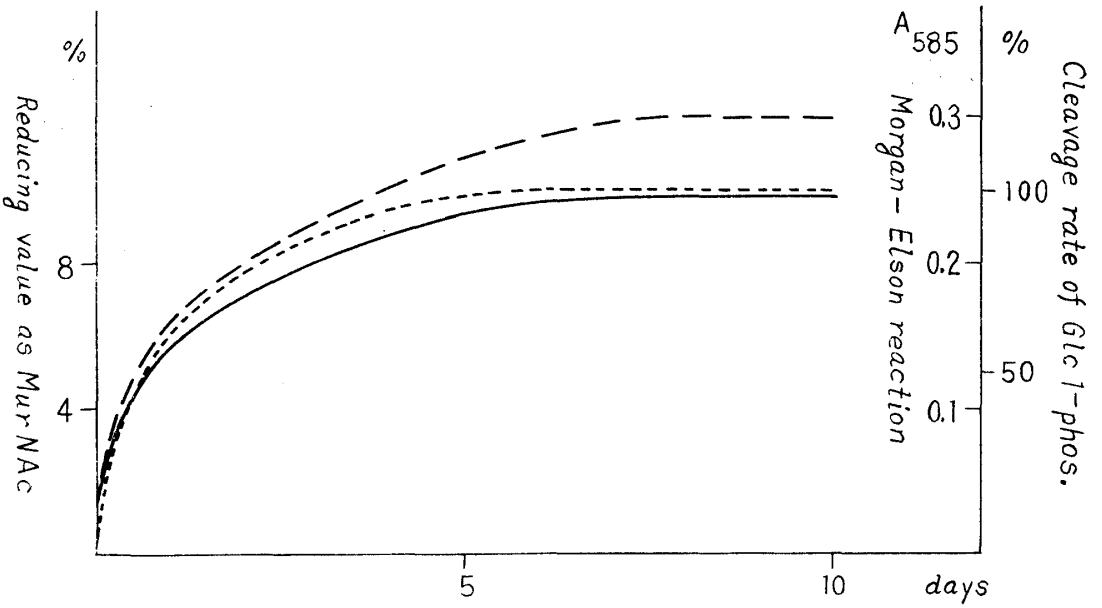

Fig. 3. Treatment with trichloroacetic acid.
—— Reducing value of the non-dialyzable fraction.
– – Coloration of the non-dialyzable fraction by Morgan–Elson reaction.
------ Cleavage rate of glucose 1-phosphate.

Treatment with Phosphatases——The trichloroacetic acid solution of the material which had been kept for 10 days at 22° was extracted thrice with equal volume of ether to remove trichloroacetic acid, then lyophilized. 5 mg of the dried sample were dissolved in 1 ml of a buffer and 20 μg of an enzyme in 50 μl of a buffer were added. The incubation was at 37° for 4 hr. and control experiments were performed without the enzyme. At the end of this period, inorganic phosphate was measured by the method of Chen, Toribara and Warner.[34] For the treatment with a wheat germ acid phosphatase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), 0.1M sodium acetate buffer, pH 5.5, was used, and 0.1M ethanolamine–hydrochloric acid buffer, pH 9.5, containing 0.05M magnesium acetate was used for a calf mucosa alkaline phosphatase. The results are given in Table VI.

38) L.F. Leloir, C.E. Cardini and J.M. Olavarria : Arch. Biochem. Biophys., **74**, 84 (1958).

TABLE VI. Liberation rates (%) of inorganic phosphate.

	acidic phosphatase	alkaline phosphatase
non-dialyzable material		
(treated with 10% TCA)	23.0	29.4
(untreated)	0.6	0.8
glucose 1-phosphate	34.2	87.6
glucose 6-phosphate	27.3	96.8

The action with a phosphodiesterase to the non-dialyzable material was examined. 20.2 mg of the sample and 200 μg of a snake venom phosphodiesterase (Ex Crotalus adamanteus venom; Sigma Chemical Co.) were dissolved in 5 ml of 0.05M sodium barbiturate buffer, pH 9.3, and incubated at 37° for 1 hr. The solution was diluted with the same volume of water, then 0.1 ml of the solution was withdrawn for the measurement of reducing value by the method of Park and Johnson[33] and 0.5 ml of the solution was withdrawn for the measurement of coloration by modified method[30] of Morgan-Elson reaction with heating of 30 min. Resulting reducing value was 3.3% as glucose and absorbance at 585 mμ by Morgan-Elson reaction was 0.172. These results show the fact that the split of a part of phosphate linkages occured by the phosphodiesterase treatment.

Fractionation of F-III after Treatment with Trichloroacetic acid——F-III, the main fraction from non-dialyzable material, was treated with trichloroacetic acid at 22° for 7 days as described above, and after removal of the acid, lyophilized product was used as the sample for the following fractionation. In addition to this condition, two other conditions, 10% trichloroacetic acid at 4° and 5% trichloroacetic acid at 35°, were also attempted. But these conditions were not used for the pre-treatment of F-III, because the treatment at 4° gave much weaker action for the cleavage of phosphate linkages, and no good result for the following purification was obtained by the treatment at 35°.

500 mg of the sample were dissolved in 10 ml of 0.1M sodium acetate and applied to a column (2.5×43 cm) of DEAE-Sephadex A50 which had been equilibrated with 0.1M sodium acetate after washing with 0.5N hydrochloric acid, then 0.5N sodium hydroxide and finally water. A mixing chamber containing 500 ml of 0.1M sodium acetate, into which was passed 550 ml of the same solvent followed by 0.5M lithium chloride in 0.1M sodium acetate from a reservoir, was attached to the column. Fractions of 10 ml were collected at a rate of 1 ml per 6 min. Aliquots of 0.5 ml were withdrawn from each fraction and analyzed by the modified method of Morgan-Elson reaction[30] and 0.1 ml for the analysis by phenol sulfuric acid method.[32] For the determination by the latter method, 0.1 ml of the sample was diluted to 1 ml with water, then added 0.025 ml of 80% phenol and 2.5 ml of sulfuric acid. The results are given in Fig. 4. The eluates obtained from tubes 18 to 21 were collected and evaporated under reduced pressure. In order to remove the coexistent salt, the residue was dissolved in small amount of water and applied to a

column (2.5×80 cm) of Sephadex G–25 and developed with water. Morgan–Elson reaction positive fractions were collected and concentrated under reduced pressure, then lyophilized. This will be termed as DS–I. Similarly, DS–II was obtained from tubes 24 to 34, DS–III from tubes 35 to 89, DS–IV from tubes 90 to 99 and DS–V from tubes 100 to 118 after removal of the salt. The yields were 15 mg in DS–I, 14 mg in DS–II, 50 mg in DS–III, 115 mg in DS–IV and 206 mg in DS–V. The values are corrected for loss by measurements.

Water solution of DS–IV and DS–V were separately applied to a column (2.6×81 cm) of Sephadex G–100 which had been equilibrated with 0.05*M* pyridine-acetic acid buffer, pH 5. The column was eluted with the same solvent at a flow rate of 10 ml per 25 min. and fractions of 20 ml were collected. Analysis by the modified method of Morgan–Elson reaction and by phenol–sulfuric acid method were carried out as described above. The results are shown in Fig. 5. The eluates obtained from tubes 8 to 13 in Fig. 5–1 were col-

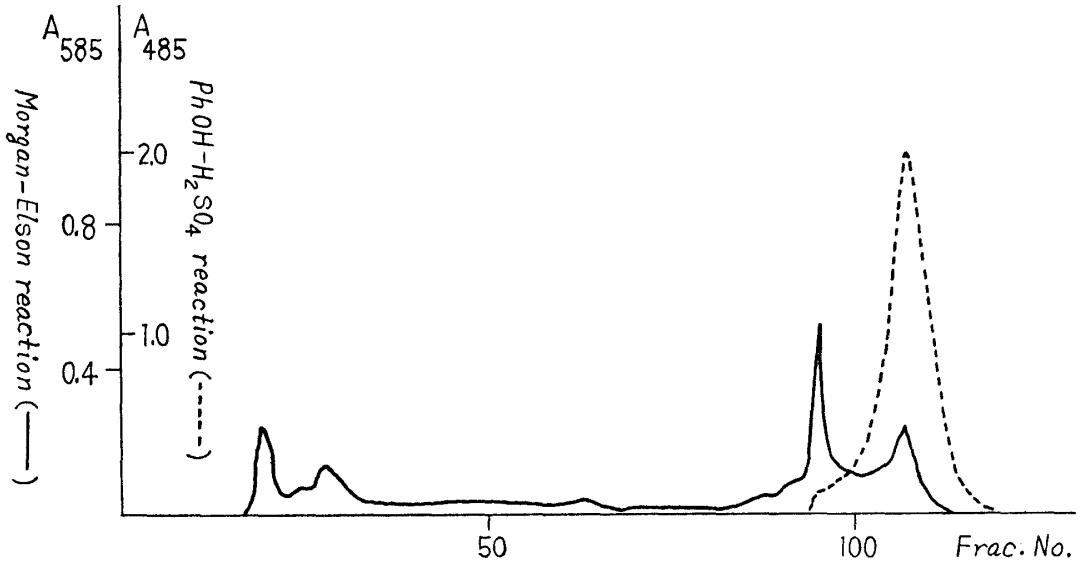

Fig. 4. Chromatography of TCA-treated F III on DEAE-Sephadex A50.

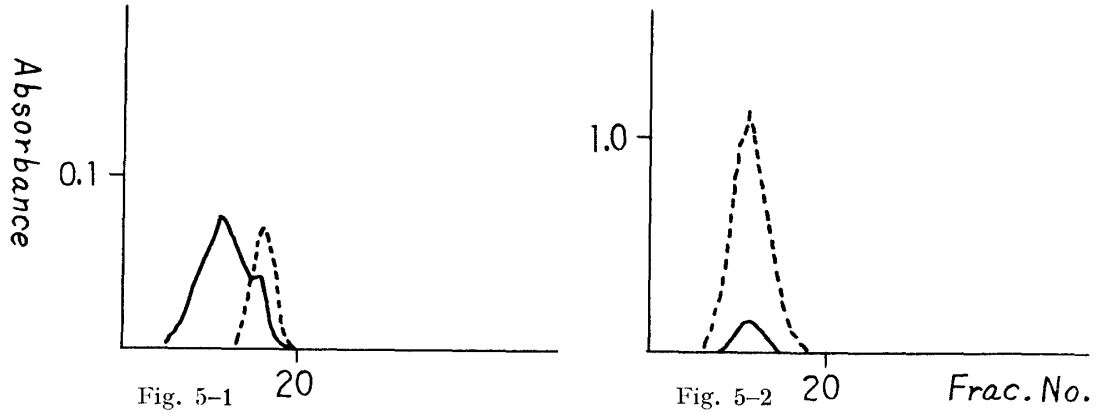

Fig. 5–1 Fig. 5–2

Fig. 5. Gel filtrations of DS–IV and DS–V on Sephadex G–100.

lected and evaporated under reduced pressure. The water solution of the residue was lyophilized. This will be termed as DS-IV-A. Simlarly, DS-IV-B was obtained from tubes 15 to 17 in Fig. 5-1 and DS-V-A from tubes 6 to 16 in Fig. 5-2. The yields were 61 mg in DS-IV-A, 23 mg in DS-IV-B and 201 mg in DS-V-A.

Properties of Fractions obtained by Ion Exchange Chromatography and Gel Filtration——DS-IV-A and DS-V-A, the two main fractions obtained by chromatography on DEAE-Sephades A50 and gel filtration on Sephadex G-100, were respectively homogeneous on thin-layer electrophoresis. The resulting distances from the starting point were 5 mm to cathodic side in DS-IV-A and 16 mm to anodic side in DS-V-A using pyridine-acetic acid buffer. Qualitative and quantitative analysis of them were carried out in the same way as the case of non-dialyzable fractions. The sugar compositions of them are given in Table VII.

TABLE VII. Sugar compositions (%) of DS-IV-A and DS-V-A.

	DS-IV-A	DS-V-A
glucose	0	30.9
muramic acid	12.8	10.7
glucosamine	11.4	10.3
muramic acid 6-phosphate	2.10	1.94
glucosamine 6-phosphate	0.28	0
phosphorus (organic)	0.17	0.15

Both substances contain alanine, glutamic acid, glycine and lysine as component amino acids in addition to the similar unknown components to those of F-III. The kinds of component sugar and amino acid of DS-I, DS-II and DS-III are similar to those of DS-IV-A, with the exception of the fact that no organic phosphate is contained in them.

Reduction with Sodium Borohydride and Borotritide——10 mg of each sample was dissolved in 0.2 ml of water and added 10 mg of sodium borohydride. After the mixture had been kept for 2 hr. at room temperature, the pH was adjusted to 5 by addition of Dowex 50W-X8, H^+form. The resins were filtered off, and after washing with 0.5 ml of water and methanol, the filtrate and washings were combined and evaporated under reduced pressure. 1 ml of methanol was added to the residue and evaporated again. This process was repeated three times.

In order to obtain a radioactive reduced product, 8 mg of sodium borohydride and 2 mg of sodium borotritide were used instead of 10 mg of sodium borohydride. After 2 hr. at room temperature, 1 ml of methanol was added and the pH was adjusted to 5 by addition of acetic acid. Then the solvent was evaporated at 40° by blowing of air in a draft chamber. 0.5 ml of methanol were added to the residue and evaporated again. The residue was dissolved in 1 ml of water and the solution was passed into a small column (2×10 mm) of Dowex 50W-X8, H^+form, then washed with 1 ml of water and methanol. The filtrate

and washings were combined and evaporated in vacuo.

Analysis of Hydrolyzates of Reduced Products——The reduced products were hydrolyzed with 4*N* hydrochloric acid at 100° for 1 hr. as described above. After removal of the HCl, the hydrolyzates were analyzed by cellulose thin-layer chromatography. Periodate-benzidine reagent[39] was used for detection. Rf values of muramicitol, glucosaminitol and sorbitol were 0.45, 0.26 and 0.36 in Solvent B, and 0.21, 0.25 and 0.41 in Solvent F. Among these three alditols, muramicitol was only one which was obviously detected from the hydrolyzates of reduced DS-IV-A, DS-V-A and F-III. The differences of relative radioactivities were almost parallel to those of reducing values before reduction in these samples, although the distribution of radioactivity was scattered even in thin-layer chromatogram of standard muramicitol. The results are shown in Table VIII.

TABLE VIII. Radioactivities of chromatograms of the hydrolyzates and reducing values of the originals.

	relative radioactivity*	reducing value**
DS-IV-A	12.8	15.0
DS-V-A	3.7	5.8
F-III	3.0	6.2

* Relative value to muramicitol (muramicitol=100).

** Reducing value (%) as N-acetyl muramic acid.

Reducing values of DS-II and DS-III were 25.3% and 17.4% as N-acetyl muramic acid, but the reduction was not applied to these fractions.

Discussion

The non-dialyzable fraction obtained from cell walls of *Micrococcus lysodeikticus* by treatment with lysozyme was purified into three fractions. By the analysis of the hydrolyzate with hydrochloric acid, it was confirmed that each fraction has two kinds of amino sugar phosphates, muramic acid 6-phosphate and glucosamine 6-phosphate, in addition to well-known amino sugar components, muramic acid and glucosamine. It is conceivable that the most of component amino sugars and amino sugar phosphates are in existence as their N-acetyl derivatives. The main differences of relative amounts of known components in these fractions were found in the amounts of glucose and peptide (Table V). Each fraction has the same pentapeptide having molar proportions of alanine 2, glutamic acid 1, glycine 1 and lysine 1. But F-I contains much smaller amount of peptide than the other two fractions and, on the other hand, the amount of glucose is larger. The variation in the amino acid content of peptidoglycan has been already observed in the Pneumococcal cell walls.[15] The fact described above also supports the considerable variation in the peptide content of another bacterial cell wall. The author chose F-III as the material for the treatment of trichloroacetic acid in respect of the yield, but F-I must be another interest-

39) J.A. Cifonelli and F. Smith : Anal. Chem., **26**, 1132 (1954).

ing material. The presence of a polysaccharide composed of glucose and 2-acetamido-2-deoxy-mannuronic acid as the component of Micrococcus lysodeikticus cell walls had been suggested by Perkins.[40] The conspicucous difference in glucose content of the fractions offers the possibility of the presence of such polysaccharide combined with peptidoglycan. The attempts of isolation, however, of the glucose-rich polysaccharide containing no peptidoglycan have not been successful.

Since very few inorganic phosphates were liberated from the non-dialyzable fraction by the treatment with a wheat germ acid phosphatase, as well as a calf mucosa alkaline phosphatase, it is conceivable that the amino sugar phosphates in the cell walls form generally phosphodiester linkages. This conclusion is also supported by the fact that a snake venom phosphodiesterase splits a part of linkages and results in some appearances of terminal reducing N-acetyl amino sugars. The similar effect to that of a phosphodiesterase was observed by treatment with trichloroacetic acid at 22°, and after the treatment, the non-dialyzable fraction produced considerable amounts of inorganic phosphate by the action of monophosphatases. 1-Phosphate linkages of N-acetylhexosamine and glucose were completely split by this treatment during 7 days. On the other hand, amino sugar 6-phosphate was completely stable. These results suggest a hypothesis that 1-phosphate linkages of phosphodiesters in the non-dialyzable fraction are split by the treatment with trichloroacetic acid.

There are several reports which described on the presences of phosphodiester linkages between peptidoglycan and the other polysaccharide or teichoic acid in bacterial cell walls. Those are, the teichoic acid-peptidoglycan complex in *Staphylococcus aureus* walls,[41] the similar teichoic acid-peptidoglycan complex in *Staphylococcus lactis* walls,[42] the linkage between peptidoglycan and the polysaccharide composed of rhamnose and N-acetyl glucosamine in *Lactobacillus casei* walls,[43] and the linkage between peptidoglycan and N-acetyl galactosamine phosphate polymer in Pneumococcal walls[15] Until recently, it had been believed that the cell wall of *Micrococcus lysodeikticus* has no organic phosphorus. But more recently, it was observed that muramic acid 6-phosphate is widely distributed in the cell walls of Gram-positive bacteria including *Micrococcus lysodeikticus* by Liu and Gotschlich.[44] Muramic acid 6-phosphate was first isolated from *Lactobacillus casei* by Ågren and de Verdier,[45] and now a suggestion that phosphorylated muramic acid residues may function as bridges between the peptidoglycan and the other polysaccharide or teichoic acid has been proposed.[42,43,44,46,57] Most recently, it was reported that segments of the

40) H.R. Perkins : Biochem. J., **86**, 475 (1963).
41) J.-M. Ghuysen, D.J. Tipper and J.L. Strominger : Biochemistry, **4**, 474 (1965).
42) D. Button, A.R. Archibald and J. Baddiley : Biochem. J., **99**, 11c (1966).
43) K.W. Knox and E.A. Hall : Biochem. J., **96**, 302 (1965).
44) T.-Y. Liu and E.C. Gotschlich : J. Biol. Chem., **242**, 471 (1967).
45) G. Ågren and C.H. de Verdier : Acta Chem. Scand., **12**, 1927 (1958).
46) H. Heymann, G.M. Manniello and S.S. Barkulis : Biochem. Biophys. Res. Comm., **26**, 486 (1967).
47) W.D. Grant and A.J. Wicken : Biochem. Biophys. Res. Comm., **32**, 122 (1968).

glycan, consisting of 8 to 18 disaccharide units, are linked through 1 muramic acid phosphate to a segment, consisting of about 10 glucose residues, of a second wall polymer in *Micrococcus lysodeikticus*, *Micrococcus flavus* and *Sarcina lutea*.[20] In addition to muramic acid 6-phosphate, glucosamine 6-phosphate was also obtained from the non-dialyzable fractions in this work. Therefore the author is albe to propose a possibility that the both amino sugar phosphates serve as bridges between a peptidoglycan and the other fraction. There is considerable difference in glucosamine 6-phosphate content of the purified non-dialyzable fractions (Table V), and a peptidoglycan which has only muramic acid 6-phosphate as a component phosphate was obtained after treatment with trichloroacetic acid (Table VII). These facts support the variation in glucosamine 6-phosphate content of peptidoglycan in the cell walls.

The all fractions isolated from trichloroacetic acid-treated F-III using DEAE-Sephadex chromatography were peptidoglycans. Several fractions have considerably higher reducing values than that of the original material and some of them possess no phosphate. It is conceivable that they were originally combined with the main peptidoglycan by their terminal reducing groups which appeared by cleavage of phosphodiester linkages, and since the increase of coloration by Morgan-Elson reaction was parallel to that of reducing value, there is considerable possibility that some N-acetyl amino sugar is the terminal reducing group. After reduction of DS-IV-A with sodium borohydride, muramicitol was detected from the hydrolyzate. From these results, it is able to propose a hypothesis that at least a part of peptidoglycans combines to the other peptidoglycans at the C-1 position of its terminal muramic acid with a phosphodiester linkage combined to C-6 position of an amino sugar in the other peptidoglycan. Unfortunately, the samples have many components which are detectable by benzidine-periodate reagent, and the distribution of the radioactivity of reduced product of muramic acid with sodium borotritide was scattered. Therefore, it is very difficult to decide whether muramicitol is the only product of the reduction or not. In addition, several unknown components have been found in the hydrolyzates of the non-dialyzable fractions (Table IV). We have still many unsolved problems. Further investigations on F-I and the fractions obtained by the ion exchange chromatography, however, may produce valuable informations on the structure of the cell wall.

Acknowledgment

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