UDC 547.963 : 576.851.252

70. Shichiro Akiya*1 and Otomatsu Hoshino*2 : Studies on the Constitution of Muco-complex from *Micrococcus lysodeikticus*. III.[1] Properties of Acid Hydrolysis Products.

(*Tokyo Medico-Dental University*1 *and Faculty of Pharmaceutical Sciences, University of Tokyo*2)

In the investigations on constituents of cell walls of *Micrococcus lysodeikticus*, Epstein, *et al.*[2] and Meyer, *et al.*[3] isolated a water-soluble mucopolysaccharide which was hydrolysed by lysozyme to give reducing substances and acetylhexosamine-like substances. Further, Hawthorne[4] reported the presence of glucose and mannose in the acid hydrolysates of the lysozyme-digest of the same bacteria.

In 1951, Salton[5,6] isolated the cell walls as a water-insoluble fraction and the latter was hydrolysed to several amino acids, glucose, and glucosamine[7] as well as a new amino-sugar[8] which was presumed to be the same as that reported by Strange and Powell[9] and by Cummins and Harris[10] in their respective studies on bacterial spore peptides and cell walls. Further, by a more detailed investigations, a structure of 3-O-(2-carboxyethyl)hexosamine was proposed for the new amino sugar[11,12] which was named muramic acid,[13] and the proposed structure was supported by comparison with synthetic 3-O-(2-carboxyethyl)-D-glucosamine.[14,15]

On the other hand, by further examinations on constituents of soluble mucopolysaccharide from *M. lysodeikticus*, Schütte and Krisch[16,17] detected glucose and glucosamine, as well as small amounts of mannose and fucose. In the first paper[18] of this series, the authors reported that water-soluble mucopolysaccharide prepared by Meyer's method was further separated by acrinol method into two fractions, fractions G and H, the former consisting of muco-complex and the latter, mainly of mannose.

Of the above-cited several constituents obtainable from *M. lysodeikticus*, mannose and the fraction H respectively reported by Krisch and the present authors seem to be produced from protoplastic carbohydrates of the bacteria, because Gilby, Few, and McQuillen[19] recently reported that mannose was the main constituent of carbohydrate fraction from the protoplast membrane of this bacteria.

---

*1 Yushima, Bunkyo-ku, Tokyo (秋谷七郎). *2 Hongo, Bunkyo-ku, Tokyo (星野乙松).

1) Part II : This Bulletin, 8, 395(1960).
2) L. A. Epstein, E. Chain : Brit. J. Exptl. Pathol., **21**, 339(1940).
3) K. Meyer, E. Hahnel : J. Biol. Chem., **163**, 723(1946).
4) J. R. Hawthorne : Biochim. et Biophys. Acta, **6**, 94(1950).
5) M. R. J. Salton, R. W. Horne : *Ibid.*, **7**, 177(1951).
6) *Idem* : Nature, **170**, 746(1952).
7) *Idem* : Biochim. et Biophys. Acta, **10**, 512(1953).
8) *Idem* : *Ibid.*, **22**, 495(1956).
9) R. E. Strange, J. F. Powell : Biochem. J., **58**, 80(1954).
10) C. S. Cummins, H. Harris : *Ibid.*, **57**, xxxii(1954); J. Gen. Microbiol., **13**, iii(1955).
11) R. E. Strange, F. A. Dark : Nature, **177**, 186(1956).
12) R. E. Strange : Biochem. J., **64**, 23(1956).
13) E. Work : Nature, **179**, 841(1957).
14) L. H. Kent : Biochem. J., **67**, 5p(1957).
15) R. E. Strange, L. H. Kent : *Ibid.*, **71**, 333(1959).
16) E. Schütte, K. Krisch : Z. physiol. Chem., Hoppe-Seyler's, **311**, 121(1958).
17) K. Krisch : *Ibid.*, **311**, 131(1958).
18) Part I. S. Akiya, O. Hoshino : Yakugaku Zasshi, **77**, 777(1957).
19) A. R. Gilby, A. V. Few, K. McQuillen : Biochim. et Biophys. Acta, **29**, 21(1958).

This paper deals with further investigations on the hydrolysates of muco-complex (GII) obtained in the previous work[1] of this series.

Experimental

Though in the preceding work,[1] the muco-complex was purified to electrophoretically and ultracentrifugally homogeneous state (GIIs), because of the small yield of the final fraction and of the fact that fraction GII, available in amounts, was electrophoretically pure enough for further degradative study, the present experiments were started from GII.

Estimation of Phosphorus, Sulfate-S, and Potassium—Total P and S were estimated after hydrolysis of 10 mg. of fraction G or GII with 2*N* HCl on a boiling water bath for 3 hr. P was determined colorimetrically[20] and S was measured gravimetrically as $BaSO_4$. K was estimated as K_2SO_4 by the usual method.

Paper Electrophoresis of Hydrolysates—GII (10 mg.) was hydrolysed with 2*N* HCl (1 cc.) on a boiling water bath for 5 hr. After removing the acid, the residue was dissolved in water (0.2 cc.) (sample A). Sample A (ca. 200 γ) was submitted to paper electrophoresis in a solvent of acetate or AcOH-pyridine buffer using Toyo Roshi No. 51, at 25 v/cm., 1.0~2.0 mA/cm., 60~120 min. After migration, spots were detected with ninhydrin or benzidine-trichloroacetic acid reagents.

Two-dimensional Combined Paper Chromatography and Electrophoresis—Sample A (ca. 500 γ) described above was spotted at the origin of paper as shown in Fig. 1, using the modified method of Kickhöfen[21] and was migrated in a solvent of BuOH-pyridine-AcOH-H_2O (20:10:2:468) (pH 5.8); 25 v/cm., 1.0~2.0 mA/cm., 50~60 min.

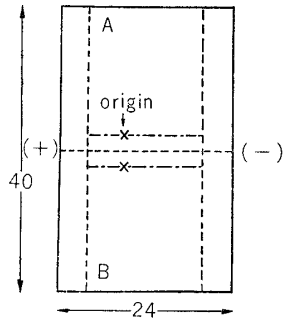


Fig. 1.

Size (cm.) of Filter Paper for Two-dimensional Paper Chromatography

After migration, the paper was dried in a current of air and submitted to paper chromatography, developed in vertical direction to that run in electrophoresis. The following solvent systems were mainly used: BuOH-pyridine-H_2O (6:4:3 or 1:1:1), BuOH-AcOH-H_2O (4:2:1 or 4:1:2), and phenol-H_2O (4:1). After development, the paper chromatogram was cut into two sheets, A and B. A was treated with ninhydrin for detection of amino acids and amino sugars, and B was used for detection of sugars and amino sugar.

Besides the reagents described above, Elson-Morgan reagent, ammoniacal $AgNO_3$, or periodate-benzidine reagent was also used for detection.

Reducing Power during Hydrolysis— Ten mg. of GII was hydrolysed with *N* HCl or 2*N* HCl (10 cc.) on a boiling water bath. The aliquots of the hydrolysates were taken at intervals and estimated for reducing power by Somogyi's method.[22]

Estimation of Glucose—Glucose was estimated by the cysteine-H_2SO_4[23] or the anthrone method,[24] as described in Part I of this series.[18]

Estimation of Glucosamine and Acidic Amino Sugar—a) Absorption spectra of Elson-Morgan coloration of hydrolysates: GII (10 mg.) was hydrolysed with *N* HCl (10 cc.) on a boiling water bath for 3 hr. Hexosamine was estimated by the modified Elson-Morgan method[25] using glucosamine-HCl as control.

20) H. H. Taussky, E. Shorr: J. Biol. Chem., **202**, 675(1953).
21) V. B. Kickhöfen, O. Westphal: Z. Naturforsch., **76**, 659~660(1952). cf. R. J. Block, E. L. Durrum, G. Zweig: "A Manual of Paper Chromatography and Paper Electrophoresis," 378(1955). Academic Press Inc., New York.
22) M. Somogyi: J. Biol. Chem., **160**, 61(1945).
23) Z. Disch, L. B. Schettles, M. Osvos: Arch. Biochem., **22**, 169(1949).
24) W. E. Trevelyan, J. S. Harrison: Biochem. J., **50**, 298(1952).
25) C. J. M. Rondle, W. T. J. Morgan: *Ibid.*, **61**, 586(1955).

b) Separation of glucosamine and acidic amino sugar by paper electrophoresis: Sample A (1 mg.) was streaked on the starting line of paper and submitted to electrophoresis as above. After migration both sides of the paper were cut off to narrow strips and they were sprayed with ninhydrin to detect the positions of neutral and basic bands. From the remaining part of the paper, the bands containing neutral and basic constituents were cut off and eluted with water. The eluates of both neutral and basic parts were then estimated by the Elson-Morgan method. The amount of basic compound was represented as glucosamine and that of neutral compound was calculated as muramic acid according to hexosamine value of Strange[11,15] (see Table II and Figs. 3 and 4).

Estimation of Amino Acids—GII (10 mg.) was hydrolysed with 4*N* HCl on a boiling water bath for 3 hr. After removal of HCl, the residue was dissolved in 0.2 cc. of water and 0.01 cc. of the solution was run on Toyo Roshi No. 50 (40×40 cm.) by the two-dimensional method using phenol-H_2O (4:1) and BuOH-AcOH-H_2O (4:2:1). After drying the paper in the air, the amino acids were detected by spraying ninhydrin reagent (ninhydrin 1 g., $CdCl_2$ 75 mg., AcOH 0.3 cc., H_2O 6 cc., made to 100 cc. with Me_2CO) by the method of Barrollier.[26] The colored spots developed after keeping the sprayed paper for 24 hr. at room temperature were cut out together with appropriate blanks and standards of known amount of amino acids. The pieces of filter paper were eluted with MeOH to make a volume of 5~10 cc. and estimated colorimetrically at 510 mμ. Similar detections of amino acids were made by the two-dimensional chromatography combined with paper electrophoresis and followed by ascending chromatography.

Dinitrophenylation of GII—To a solution of 100 mg. of GII and 100 mg. of $NaHCO_3$ in 5 cc. of water 0.1 cc. of DNFB and 5 cc. of EtOH were added. After shaking the mixture occasionally for 3 hr. in the dark, AcOH was added to the reaction mixture to bring pH to 5.0 and DNP-GII formed was precipitated by addition of EtOH (10 cc.). The precipitate was centrifuged, washed with EtOH and Et_2O, and dried *in vacuo* to pale yellow powder, ca. 90 mg.

Absorption Curve of DNP-GII—DNP-GII was dissolved in 1% solution of $NaHCO_3$ and the absorption measured in the range of 300~400 mμ. 1% $NaHCO_3$ solutions of DNP-Ala and DNP-Gly were used as controls.

Paper Chromatography of Hydrolysate of DNP-GII—DNP-GII (10 mg.) was hydrolysed with 4*N* HCl (1 cc.) on a boiling water bath for 3 hr. On extraction of the hydrolysate with Et_2O and removal of the solvent from Et_2O layer, no detectable DNP-amino acid but small quantity of dinitrophenol was found in the residue. From the aqueous layer HCl was removed by repeated evaporation of water *in vacuo* and the final concentrate was subjected to combined paper electrophoresis and paper chromatography as described above. ε-DNP-Lys was detected in the neutral portion.

Results and Discussion

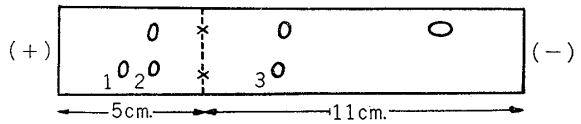
As shown in Table I the results of elemental analysis showed that both fractions G and GII contain negligible amounts of phosphorus and sulfur which seemed not to be the component of the original muco-complex. The amount of nitrogen was consistent with the amount calculated for amino acids and animo sugars contained in the muco-complex, as will be described later.

Table I. Elemental Analysis of Fractions G and GII

	Fraction			Fraction	
	G (%)	GII (%)		G (%)	GII (%)
C	41.25	40.41	Total P	0.51	0.26
H	6.09	5.59	Hydrolysable S	1.25	0.50
Total N	5.24	6.64	Ash (K)	7.94	6.82

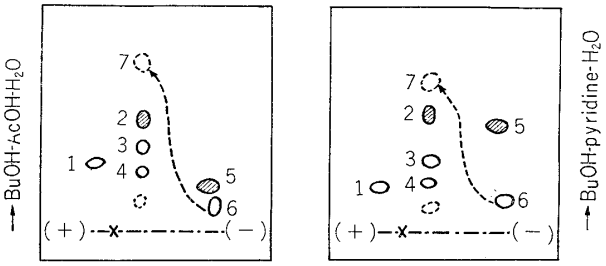
The paper electrophoretic pattern of the acid hydrolysate of GII is given in Fig. 2. Thus the hydrolysate was separated into three spots, acidic, neutral, and basic. Because no further separation of these three spots by several alternative conditions tested was achieved by one-dimensional phoresis, the pattern obtained was further submitted to paper chromatography. As shown in Fig. 3 the acidic spot gave only one component, glutamic acid, the basic spots were separated into lysine and a hexosamine, and the neutral spots were further analysed to give alanine, glycine, a hexose, and an amino sugar.

26) J. Barrollier: Naturwissenschaften, **14**, 416(1955).



spot 1 : DNP-glutamic acid
〃 2 : DNP-glycine
〃 3 : ε-DNP-lysine

Fig. 2. Paper Electrophoresis of Hydrolysate of G II



1. Glutamic acid
2. Amino sugar
3. Alanine
4. Glycine
5. Glucosamine
6. Lysine
7. ε-DNP-lysine

(25 v/cm., 60 min.)

Fig. 3. Two-dimensional Chromatograms of Combined Paper Electrophoresis with Ascending Chromatography

By both Dische's cysteine-sulfuric acid method and two-dimensional combined chromatography, the hexose was identified as glucose. Further, the hexosamine was identified as glucosamine which was also reported by Salton[7,8] and Krisch.[17] The spot of acidic amino sugar moving in neutral part was positive to benzidine-trichloroacetic acid and ammoniacal silver nitrate reagents. As this spot behaved as a neutral compound on paper electrophoresis, it was assumed to contain both free amino and carboxylic groups as Salton[22] had pointed out.

The acid hydrolysate of GII was submitted to the modified Elson-Morgan reaction and absorption curves of the resulting colored solution (Fig. 4) showed some differences with that of pure glucosamine with a maximum absorption at ca. 530 mμ. After separation of the acid hydrolysate by paper electrophoresis, the resulting neutral fraction and the basic fraction gave respective absorption curves II and III (Fig. 4) for Elson-Morgan coloration. Curve II with a maximum at 510 mμ corresponded to the similar curve for muramic acid isolated by Strange, *et al.*[11,15] and curve III for the basic component with that of glucosamine (maximum absorption at 530 mμ). Thus the curve I obtained for the hydrolysate of GII was concluded to be a synthesized curve of curves II and III, and that this hydrolysate contained both muramic acid and glucosamine in a ratio of approximately 1:1.

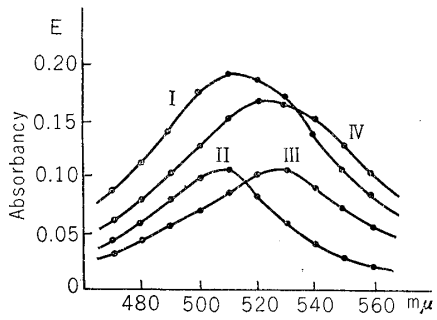


Fig. 4.
Absorption Spectra of Chromogens produced by Elson-Morgan Reaction

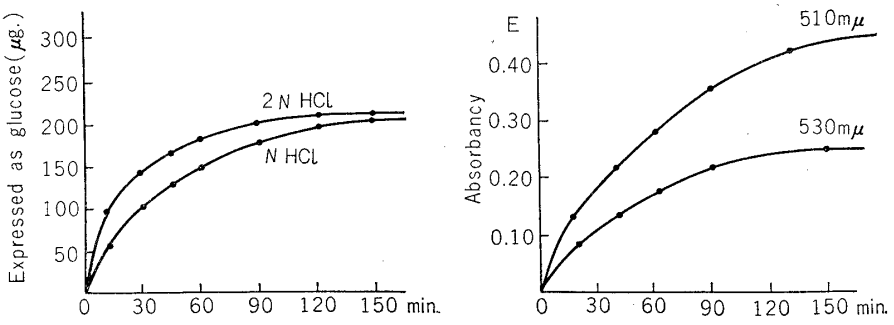
I : Hydrolysate of fraction GII
II : Neutral part obtained after paper electrophoresis
III : Basic part obtained after paper electrophoresis
IV : Glucosamine for control

The muramic acid-like compound described above was compared with several known acidic amino sugars, D-glucosaminuronic acid[27] and neuraminic acid by paper chromatography, but no identity was revealed with these compounds. From the results of

27) K. Heyns, H. Paulsen : Ber., 88, 188(1955).

absorption curve of the Elson–Morgan reaction of the neutrally migrating amino sugar and good agreement of Rf values of this compound with muramic acid*[3] in serveral different solvent systems,[15,28,29] this amino sugar was confirmed to be muramic acid.

Fig. 5 shows the increasing reducing power and liberation of amino sugar in acid hydrolysis of GII by Somogyi's and Elson–Morgan's methods, respectively.



a) Change of Reducing Power during Hydrolysis of GII

b) Change of Absorbancy at 510 and 530 mμ in Elson–Morgan Reaction during Hydrolysis of GII

Fig. 5.

Table II. Quantitative Analysis of Constituents of Fraction GII

Reducing substances (expressed as glucose)	42.6 (%)	Alanine	6.23 (%)
Glucose (anthrone)	13.6	Glycine	3.15
Glucosamine	19.1	Glutamic acid	8.82
Amino sugar (as MA)	26.6	Lysine	4.67
Acetyl	12.5		22.87
	71.8		

The result of quantitative analysis of constituents of GII is summarized in Table II.

After reaction of GII with 2,4-dinitro-1-fluorobenzene, 1% sodium hydrogencarbonate solution of resulting DNP-GII was submitted to the estimation of absorption at 360 mμ. The result indicated that the muco-complex, GII, has one free paimary amino group for each molecular weight of 2,800~3,200.

The acid hydrolysate of DNP-GII, on two-dimensional paper chromatography, gave no free lysine but ε-DNP-lysine as shown in Fig. 3 and no other DNP-amino acid.

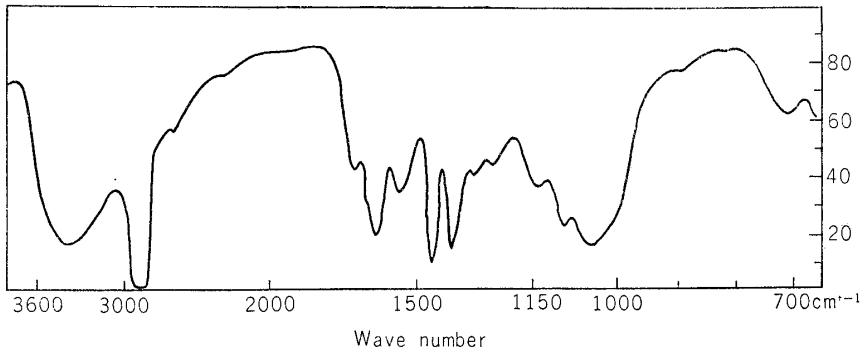


Fig. 6. Infrared Absorption Spectra of Fraction GII

*[3] The authentic specimen of muramic acid was kindly supplied by Dr. R.E. Strange of Microbiological Research Establishment, Porton, England.

28) J.T. Park: J. Biol. Chem., **194**, 877, 885(1952).
29) J.T. Park, J.L. Strominger: Science, **125**, 99(1957).

From these results the peptide constituting the muco-complex, GII, is most likely to have no primary amino group other than that in ε-position of lysine. Thus the N-terminal amino group of the peptides is assumed to combine with the sugar.[29,30]

The infrared spectrum of GII gives no special absorptions other than those corresponding to the constituent sugars and amino acids (Fig. 6).

The authors wish to express their deep appreciations to Prof. T. Ukita and Assist. Prof. S. Okui for their helpful advices and encouragements. The authors wish to thank Prof. Dr. R. E. Strange of Microbiological Research Establishment, Porton, England, who very kindly provided the authentic specimen of muramic acid for identification. They also thank Assist. Prof. T. Yamakawa of the Institute of Infectious Diseases for the supply of sialic acid, Mr. T. Nagai and Mr. Y. Oba for their helpful cooperations, to the members of Central Analysis Room for elemental analyses, and to Mr. K. Tanikawa for infrared spectral data.

Summary

The hydrolysate of muco-complex fraction (GII), isolated from *Micrococcus lysodeikticus*, was submitted to combined paper electrophoresis and paper chromatography, and was found to contain glucose, glucosamine, alanine, glycine, glutamic acid, lysine, and muramic acid. The last one was identified with the authentic specimen of muramic acid. The nature of the primary amino group of its peptide parts was investigated by dinitrophenylation of the muco-complex. Further, several components of the complex were quantitatively analysed.

(Received September 23, 1959)

30) V. M. Ingram, M. R. J. Salton : Biochim. et Biophys. Acta, **24**, 9(1957).