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Lysis of *Staphylococcus aureus* Cell Walls by a Lytic Enzyme Purified from Culture Supernatants of *Flavobacterium species*.*

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SUMMARY

1. Culture supernatants of *Flavobacterium sp.* grown in 0.1 per cent casamino acid medium for several days at 30°C were found to exhibit a marked lytic activity against intact cells and isolated cell walls of *Staphylococcus aureus*.

2. The factor responsible for this cell wall lytic activity was concentrated from the culture supernatants by precipitation with $ZnCl_2$ or $(NH_4)_2SO_4$ and purified by chromatography on a hydroxylapatite column.

3. The lytic factor is non-dialyzable through cellulose tubing, although some decrease in its activity was observed during dialysis for some unknown reason. It was inactivated completely by heating at 60°C for 60 minutes at a neutral pH. The optimum pH for its lytic activity was found to be around 6.5.

4. The activity of the lytic factor was almost completely inhibited by 0.00033 M diisopropylphosphofluoridate and 1 M NaCl. No stimulation of the lytic activity was seen with any of divalent metal ions tested. Definite inhibition of the activity, on the other hand, was effected by Zn^{++}, Cu^{++} and Cd^{++}. The findings described in this and foregoing paragraphs clearly indicate that the cell wall lytic factor concerned in this study is a type of enzyme (designated as L_{11} enzyme).

5. As regards its lytic activity range, L_{11} enzyme was found to be active against *Micrococcus lysodeikticus* as well as *Staphylococcus aureus*. However the cell walls of *Corynebacterium diphtheriae*, *Streptococcus pyogenes* (Group A) and BCG were hardly affected by this enzyme.

6. About half (by weight) the *Staphylococcus aureus* cell walls were shown to be converted into dialyzable fractions under the action of L_{11} enzyme. It was demonstrated by quantitative chemical analysis that lysis of the cell walls or a mucopeptide derived from them by L_{11} enzyme was accompanied by the release of a considerable amount of materials which, in an unhydrolysed state, reacted with ninhydrin but showed only a slight reducing activity. Lysis of the mucopeptide by egg white lysozyme, on the other hand, resulted in the liberation of reducing compounds giving a positive hexosamine reaction. Analysis by paper chromatography indicated that dialyzable portion of the lysates consisted mainly of two kinds of peptides, one of which contained glutamic acid, lysine, alanine and glycine and the other only glycine. The non-dialyzable portion was found to contain glucosamine, muramic acid and material of high phosphorus content as a main constitutent.

7. The possible mechanism of cell wall lysis by L_{11} enzyme is discussed.

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* A part of this work was reported at the 33rd Annual Meeting of the Japan Bacteriological Society (July, 18-20, 1960, at Sapporo) and at the 35th Annual Meeting of the same Society (April, 1-3, 1962, at Nagoya).

INTRODUCTION

In a short communication from this laboratory (Kotani *et al.*, 1959c) a preliminary description was given of the general properties of a staphylolytic enzyme produced by a *Flavobacterium sp.* (designated as L_{11} bacterium) isolated from a soil sample according to the method of Salton (1955).

Studies have been continued to develop a method for purification of this lytic enzyme and to elucidate its mechanism of lysis of *Staph. aureus* cell walls. The present paper describes the results of these studies.

MATERIALS AND METHODS

1. L_{11} bacterium producing the lytic enzyme

The isolation of this bacterium was described previously (Kotani *et al.*, 1959b). The organism was maintained by serial three day-subculture at about 30°C on 0.1 per cent Bacto-peptone agar slants of the following composition: Bacto-peptone 0.1 per cent, K_2HPO_4 0.025 per cent, $MgSO_4 \cdot 7H_2O$ 0.025 per cent and Bacto-agar 1.5 per cent, pH 7.2.

2. Culture medium for production of the lytic enzyme

Three kinds of culture medium were tested for their ability to support the growth of L_{11} bacterium and for their suitability for the production of the lytic enzyme by this bacterium. The media tested were as follows: 1) a 0.1 per cent casamino acid medium (CA medium, Bacto-casamino acids, technical grade, 0.1 per cent, K_2HPO_4 0.025 per cent and $MgSO_4 \cdot 7H_2O$ 0.025 per cent; 2) modified McCarty's medium (Mori *et al.*, 1960); and 3) modified Ghuysen's medium (Mori *et al.*, 1960). The pH of all these media was adjusted to 7.2 and they were sterilized by autoclaving at 120°C for 15 minutes.

3. Preparation of cell walls of test organisms

Cell walls of *Staph. aureus* (strain Newman 1), *Streptococcus pyogenes*, Group A (strain 089), *Corynebacterium diphtheriae* (strain Toronto-Harvard, a substrain of Park-Williams No.8) and BCG (strain Takeo) were prepared according to the method previously described by Kotani *et al.*, (1959a, b) except that a Kubota 10 Kc sonic oscillator, Model KMS-100, was used in place of a Raytheon 9 Kc magnetostriction oscillator, Model S-102A, for disruption of the bacteria. A cell wall preparation of *Micrococcus lysodeikticus* (strain 2665) was isolated from cells, grown on a nutrient agar supplemented with 0.2 per cent glucose at 37°C for 48 hours, in the same way as in the preparation of *Staph. aureus* cell walls.

4. Hydroxylapatite for column chromatography

Hydroxylapatite was prepared by the method of Tiselius (1956). Fine particles which would prevent passage of solvents were separated from larger particles by decantation and discarded. The suitably sized hydroxylapatite particles thus obtained were equilibrated with 0.01 M Na-phosphate buffer at pH 6.8 by repeatedly washing them with this buffer.

5. Measurement of lytic activity

A suspension of cells or cell walls of test organisms which gave an optical density of approximately 0.3 when diluted 10-fold with distilled water was used to test the lytic activity of enzyme preparations. In the case of *Staph. aureus*, this stock suspension of cell walls contained about 7.5 mg dry weight of material/ml.

To test tubes of 18 ± 0.5 mm external diameter and 185 mm length, containing 0.4 ml of a stock suspension of the cells or cell walls to be examined, were added 2.0 ml of an appropriate dilution of

test enzyme preparation and 1.6 ml of 0.05 M Tris-HCl buffer, pH 7.2. Two controls were set up: one contained no enzyme and the other no substrate. The tubes from the test and control series were incubated at 37°C in a water bath and the change in optical density was followed in a Hitachi photoelectric colorimeter, Model EPO-B, using a No. 55 filter with maximal absorption at 550 mμ.

The figures presented as "per cent optical density reduction" in the accompanying tables and figures were calculated as follows:

$$\text{Per cent optical density reduction} = \left(1 - \frac{\text{corrected optical density at specified time}}{\text{corrected optical density at 0 time}}\right) \times 100$$

where the correction was made by substracting the optical density given by the control tube containing no substrates from that of the tubes containing both enzyme and substrate. One lytic activity unit was defined as the amount of enzyme capable of producing 50 per cent optical density reduction of a standardized cell wall suspension of *Staph. aureus* after 60 minute incubation at 37°C under the experimental conditions described above.

6. Preparation of cell wall mucopeptide from Staph. aureus

A mucopeptide fraction was prepared from *Staph. aureus* cell walls by the method of Armstrong *et al.* (1958) in the following way: a specimen of 1061 mg of *Staph. aureus* cell walls was exhaustively extracted four times, for 24 hours each time, with 32 ml portions of 10 per cent trichloroacetic acid solution at 4°C. The residue was washed twice with distilled water and dried *in vacuo* over P_2O_5. The resulting residue weighed 740 mg and represented 69.8 per cent of the original cell walls. It was a mucopeptide fraction. The extracts obtained above were combined and mixed with 2 volumes of acetone. After standing the mixture overnight in a cold room, a precipitate was collected by centrifugation at 13,000 *g* for 20 minutes, washed once with acetone and dried *in vacuo* over P_2O_5 (168 mg, 15.8 per cent of the original cell walls, a teichoic acid fraction). The supernatant fluid after separation of the precipitate gave a further precipitate on addition of acetone equivalent to 5 volumes of the starting extract (61 mg, 5.7 per cent).

7. Analytical methods

Determination of nitrogen, phosphorus, reducing sugars and ninhydrin-positive substances were performed by the methods of Yokoi and Akashi (1955), Fiske-Subbarow (Fister, 1950), Nelson (1944) and Moore and Stein (1948), respectively.

The protein nitrogen content was measured as the difference between the nitrogen content of the untreated specimen and the trichloroacetic acid (0.4 M final concentration) soluble part of it. The nitrogen content was determined by the colorimetric method of Lowry *et al.* (1951).

Hexosamines were determined by the method of Neuhaus and Letzring (1957) on a specimen hydrolyzed with 4 N HCl at 100°C for 4 hours and dried *in vacuo* over P_2O_5 and NaOH to remove HCl. The method of Leissig, Strominger and Leloir (1955) was followed in determination of the N-acetylhexosamine content, except that 0.1 M K-tetraborate was used in place of 0.8 M K-tetraborate and samples were heated in the tetraborate solution for both 3 and 60 minutes in order also to measure bound N-acetylhexosamines simultaneously (*cf.* Ghuysen and Salton, 1960).

Differential estimation of glucosamine and muramic acid was done by the method of Park, as described in the report of Perkins and Rogers (1959): a specimen was separated into glucosamine and muramic acid fractions by passing it through a column prepared from a slurry of equal parts of Norit (Norit Extra, N.V. Norit-Vereeniging, Holland) and Celite 535 (Johns-Manville Products, U. S. A.). The hexosamine content of each fraction was measured by the method of Neuhaus and Letzring. Muramic acid is known to give 27 per cent of the colour of glucosamine.

8. Paper chromatography for detection and identification of amino acids, peptides and sugars

Paper chromatography was performed at 28°C on 40 cm × 40 cm sheet of Toyo Roshi No. 51 or 51A filter paper (Toyo Roshi Co., Tokyo). The developing solvents used were as follows (figures in brackets represent ratios of volumes): *n*-butanol-acetic acid-water (3:1:1), *n*-butanol-pyridine-water

(6:4:3), *n*-butanol-pyridine-acetic acid-water (60:40:30:3), phenol-water-ammonia (80:40:0.3) and pyridine-water (4:1). Both ascending and descending procedures were employed. The amino acids and peptides were detected by spraying the dried chromatograms with 0.2 per cent ninhydrin in acetone. Reducing substances were detected by the $AgNO_3$ method of Trevelyan (1950) and the aniline hydrogen phthalate method of Partridge (1949). Hexosamines were detected by the Elson and Morgan method described by Partridge (1948). For comparison, appropriate authentic specimens of amino acids and sugars were run on the same chromatograms as the test specimens.

9. Electron microscopic observation

The techniques adopted were essentially as described by Kotani *et al.* (1959a).

RESULTS

1. *Production and preparation of the lytic enzyme*

1) *Production of the lytic enzyme by L_{11} bacterium*

Preliminary experiments on the relative merits of the culture media described above showed that CA medium was the most satisfactory. Both the modified McCarty's and Ghuysen's media allowed good growth of L_{11} bacterium, but the production of the lytic factor was poor in these media.

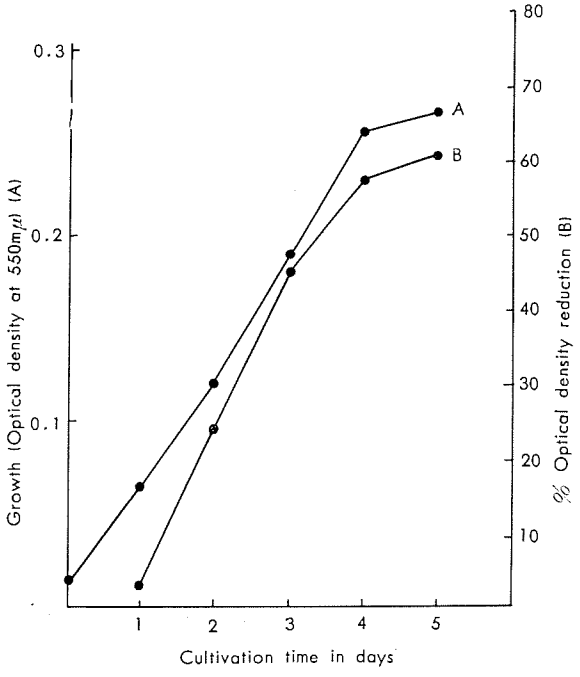


Fig. 1. Relationship between Growth of L_{11} Bacterium and Production af the Lytic Factor Active against *Staph. aureus* Cell Walls (Reproduced from Kotani *et al.*, 1959c)

A stock culture of L_{11} bacterium was grown for 3 days at 30°C on 0.1 per cent

Bacto-peptone agar slants. A standard loopful was then inoculated into 330 ml of CA medium as a shallow layer in a 1500 ml Roux's bottle. After 3 days incubation at 30°C, 10 ml aliquots of this seed culture were inoculated into 330 ml portions of the same medium for production of a large quantity of lytic culture supernatant. Fig. 1 illustrates a representative experiment on the rate and extent of the growth of L_{11} bacterium (curve A) and production of the lytic factor active against *Staph. aureus* cell walls (curve B). Active growth of L_{11} bacterium was shown to be accompanied by production of the lytic factor. The optical density of the cultures usually decreased after the 6th day, possibly as a result of autolysis of the cells, but the lytic activity of the culture supernatants was maintained at the same level for at least 3 or 4 days further. The lytic activity against *Staph. aureus* cell walls of culture supernatants obtained from a 5 to 7 day old growth of L_{11} bacterium was found to be between 1 and 3 lytic units/ml.

2) *Concentration of the lytic factor from culture supernatants*

A. *Concentration with $ZnCl_2$*

A 1.4 l portion of culture supernatant from a 4 to 5 day old culture of L_{11} bacterium was added drop by drop with 50 g/100 ml solution of $ZnCl_2$ to a final concentration of 0.025 per cent (the pH of the mixture decreased from about 8.1 to nearly 6.0). The mixture was left standing for 20 minutes at room temperature and the resulting precipitate was separated from the supernatant fluid by decantation and centrifugation. To the precipitate was added 40 ml of 20 per cent Na_2HPO_4 solution. The mixture was stood at 37°C in a water bath for 60 minutes and centrifuged to separate a dark brown solution from the white precipitate. The precipitate was extracted again with 40 ml of Na_2HPO_4 solution to recover the lytic factor as completely as possible. The two supernatants were combined and dialyzed in seamless cellulose tubing (Visking Co., U.S.A.) against distilled water for 48 hours in the cold room (designated as $ZnCl_2$-concentrate, 120 ml). About 12-fold concentration was effected by this procedure with about 70 per cent recovery (corrected for the decrease in lytic activity during dialysis, see section 2-3), but without any appreciable increase of specific activity.

B. *Concentration with $(NH_4)_2SO_4$*

Twenty l of a 5 to 7 day old culture of L_{11} bacterium in CA medium were brought to 0.3 saturation of $(NH_4)_2SO_4$ by adding 3520 g of powdered $(NH_4)_2SO_4$ and 10 g Celite 535 slowly with stirring. After standing overnight in a cold room, the mixture was filtered through filter paper in a Buchner's funnel to remove the precipitate containing the bulk of the bacterial cells. The filtrate was brought to 0.9 saturation of $(NH_4)_2SO_4$ by slow addition of 449 g of powdered $(NH_4)_2SO_4$ and 0.1 g Celite 535 per l of filtrate. The suspension was allowed to stand in the cold room overnight. The resulting dark brown precipitate was collected on filter paper in a Buchner's funnel using suction and then suspended in 300 ml of 0.01 M Na-phosphate buffer of pH 6.8. The suspension was filtered through a sintered

glass filter (3G4, Iwata Co., Osaka) to remove Celite and insoluble materials and the filter cake was washed with a small amount of the buffer. The combined filtrate and washings were saturated with powdered $(NH_4)_2SO_4$. After standing overnight in the cold room, the resultant precipitate was collected by centrifugation and dissolved into 100 ml of 0.01 M Na-phosphate buffer of pH 6.8. The solution, after filtration through a sintered glass filter, was dialyzed against a large quantity of the buffer and then against distilled water, for 24 hours each, in the cold room (designated as $(NH_4)_2SO_4$-concentrate, 110 ml). The concentrate thus obtained was shown to contain 30 to 70 per cent of the lytic activity of the starting material.

3) *Fractionation by hydroxylapatite column chromatography*

A 3 cm × 16 cm column of hydroxylapatite was equilibrated with 0.01 M Na-phosphate buffer (pH 6.8) by running 2 l of the buffer through the column. Then 45 ml portion of the (NH_4) SO_4-concentrate which had been dialyzed against 0.01 M Na-phosphate buffer for 48 hours was applied under slight pressure. Stepwise elution was performed with 1200 ml each of 0.01 M, 0.02 M and 0.05 M Na-phosphate buffer (pH 6.8) successively, at a flow rate of 20 ml/hour. Fractions of 10 ml were collected and assayed for their lytic activity against *Staph. aureus* cell walls and the absorption of their protein constituents at 280 mμ and of coloured materials at 550

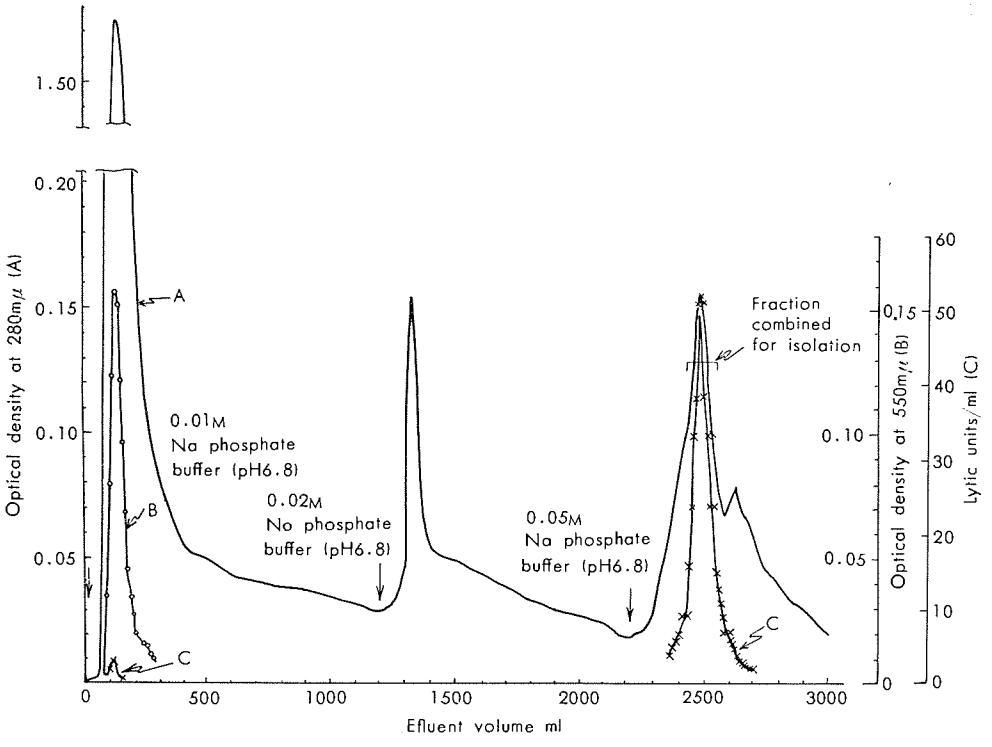


Fig. 2. Purification of the Lytic Factor by Chromatography with a Hydroxylapatite Column

mμ. As shown in the elution curve presented in Fig. 2, the bulk of the lytic factor (60 to 90 per cent in varying experiments) was eluted with 0.05 M buffer, while the effluents with 0.01 M and 0.02 M buffer contained mostly inactive proteins and coloured materials and only traces of the lytic factor. For isolation of the lytic factor, the fractions indicated in Fig. 2 were combined and freed from salts by dialysis against distilled water (purified L_{11} enzyme).

Table 1 presents the result of a representative concentration and purification experiment in terms of the percentage recovery and purification at each step. An increase in specific activity (lytic units/mg protein N) of about 6-fold was achieved in this experiment with about 20 per cent overall recovery. The specific activity of the purified fractions ranged from 2,000 to 4,000 units/mg protein N in various experiments.

Table 1. Summary of Concentration and Purification Procedures

Fraction	Volume	Lytic activity units/ml	Total lytic activity units	Protein μgN/ml	Total protein mg N	Specific activity units/mg protein N	Purification	Recovery of activity per cent
Culture supernatant	19,460	2	38,920	4.7	91.46	425	1	100
$(NH_4)_2SO_4$-concentrate (0.3-0.9 sat. fraction)	110	120	13,200	137.0	15.07	875	2.05	33.8
Fraction purified by column chromatography*	281	30	8,430	11.3	3.18	2655	6.24	21.4

* See Fig. 2.

2. *General properties of the lytic factor*

The experiments presented in this section were performed with specimens of the culture supernatant or $ZnCl_2$-concentrate, with the exception of the experiment on the influence of ionic strength on lytic activity.

1) *Optimum pH*

The lysis of *Staph. aureus* cell walls by a specimen of the $ZnCl_2$-concentrate was assayed in reaction mixtures of varying pH's, as shown in Fig. 3. Tris-maleate buffer of 0.02 M final concentration, was used in place of Tris-HCl buffer, since the former covers a wider pH range than the latter (Gomori, 1955). The optimum pH for the lytic activity of the concentrate was found to be about 6.5.

2) *Heat stability of the lytic factor*

A specimen of the $ZnCl_2$-concentrate was divided into three portions and the pH of two of them was adjusted to 4.0 and 8.0 by addition of a small amount of 0.1 N HCl or 0.1 N NaOH, respectively. Aliquots of these were heated at 60°C and 80°C for 60 minutes. The pH was readjusted to 7.2 and their lytic activities, together with that of an unheated specimen, were assayed against *Staph. aureus* cell

walls. Fig. 4 shows that the lytic factor was completely inactivated by exposure to temperatures of 60°C or more, irrespective of the pH of the medium.

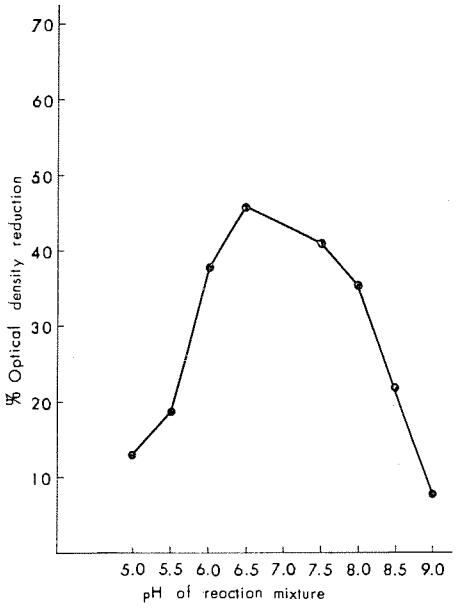


Fig. 3. The pH Optimum for the Activity of the Lytic Factor

Tris-maleate buffer was used at a final concentration of 0.02 M.

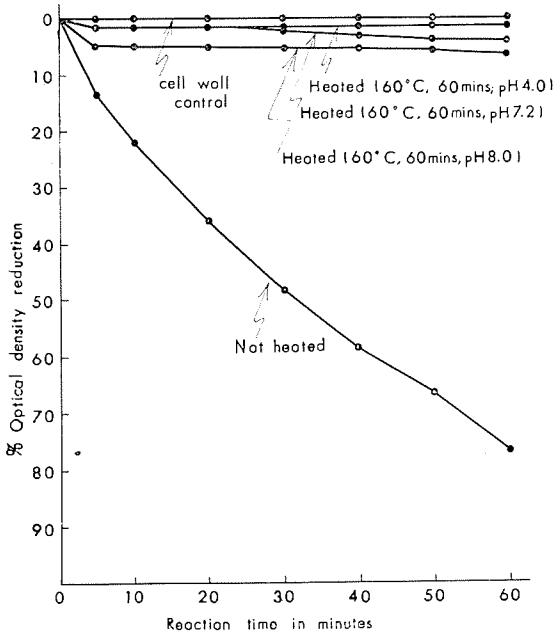


Fig. 4. Heat-Stability of the Lytic Factor

3) *Influence of dialysis upon the lytic activity*

A specimen of the culture supernatant was dialyzed in seamless cellulose tubing against a large quantity of distilled water for 48 hours in a cold room. The lytic activity of the dialyzed specimen was assayed against *Staph. aureus* cell walls. The activity of the dialyzable fraction, which was obtained by dialyzing another specimen of the same culture supernatant against an equal quantity of distilled water for 48 hours in the cold, was also measured. It will be noted from Fig. 5 that the

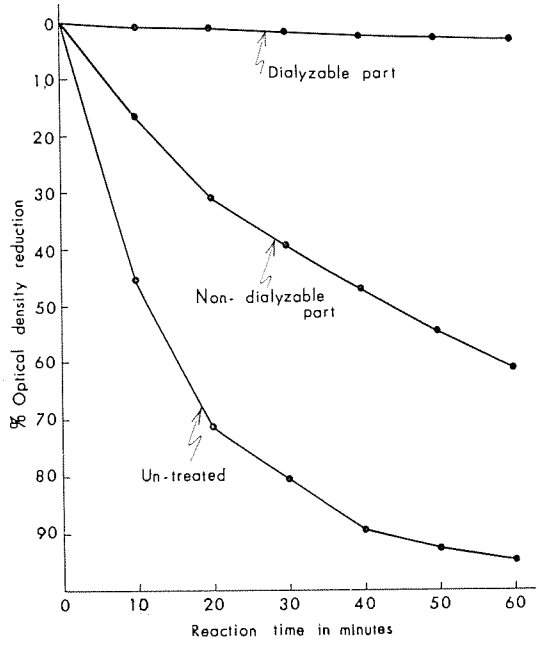


Fig. 5. Effect of Dialysis on the Activity of the Lytic Factor

cell wall lytic activity of the culture supernatants decreased significantly on dialysis but that the lytic activity was not found in the dialyzable portion. It may be added in this connection that a specimen of the culture supernatant, heated at 100°C for 20 minutes, significantly restored the lytic activity of the dialyzed specimen. The nature of this activation, however, is not yet known.

4) *Inhibitors of the lytic factor*

In view of the observations presented above, the possible activating or inhibiting effects of divalent cations (at a final concentration of 0.001 M) upon the cell wall lytic activity of the $ZnCl_2$-concentrate were studied as shown in Fig. 6. None of the divalent cations tested had an activating effect and Zn^{++}, Cu^{++} and Cd^{++} strongly inhibited the lytic factor.

Fig. 7 presents the results of an experiment on the influence on the lytic activity of the $ZnCl_2$-concentrate of a variety of compounds known to inhibit various

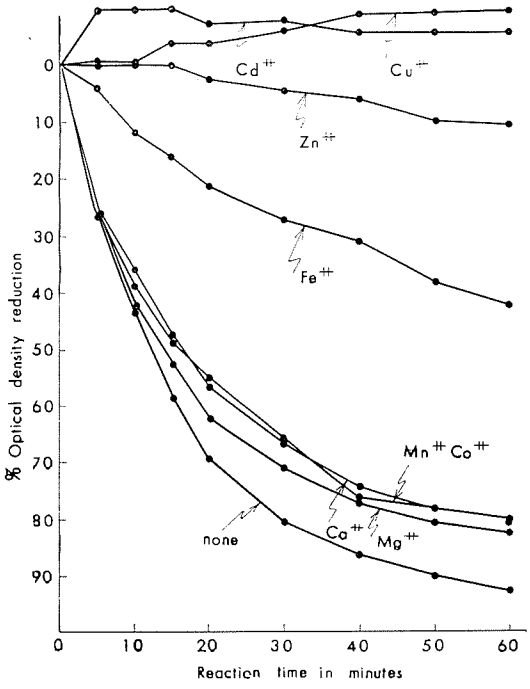


Fig. 6. Inhibitory Effect of Divalent Cations on the Activity of the Lytic Factor

All divalent cations tested were added to the reaction mixtures at a final concentration of 0.001 M.

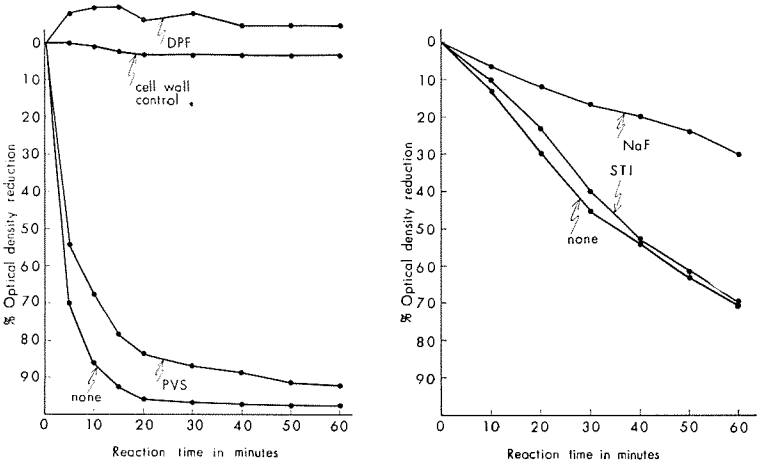


Fig. 7. Effect of Enzyme Inhibitors on the Activity of the Lytic Factor

DFP : Diisopropylphosphofloridate (0.00033 M); PVS : Polyvinylsulfate (0.25 per cent); NaF (0.05 M); and STI : Soy bean trypsin inhibitor (100 μg/ml).

enzymes. The inhibitors tested were as follows: diisopropylphosphofluoridate, 0.00033 M; polyvinyl sulfate (an inhibitor of ribonuclease and other enzymes, Nomura *et al.*, 1958), 0.25 per cent; NaF, 0.05 M; and soy bean trypsin inhibitor (Nutritional Biochemicals Corporation, U.S.A.), 100 μg/ml (all figures cited above represent final concentrations of the compounds in the reaction mixture). It will be seen that diisopropylphosphofluoridate exhibited a marked inhibitory effect upon the activity of the lytic factor, NaF had a slight effect and the other two compounds tested had no inhibitory effect.

5) *Influence of the ionic strength of the assay medium on the lytic activity*

The influence of addition of various concentrations of NaCl to the assay medium on the lytic activity of a purified L_{11} enzyme was studied. The concentration of Tris-HCl buffer in the mixture was 0.01 M. The lytic activity of the enzyme was almost completely inhibited by over 0.5 M NaCl, as indicated in Fig. 8.

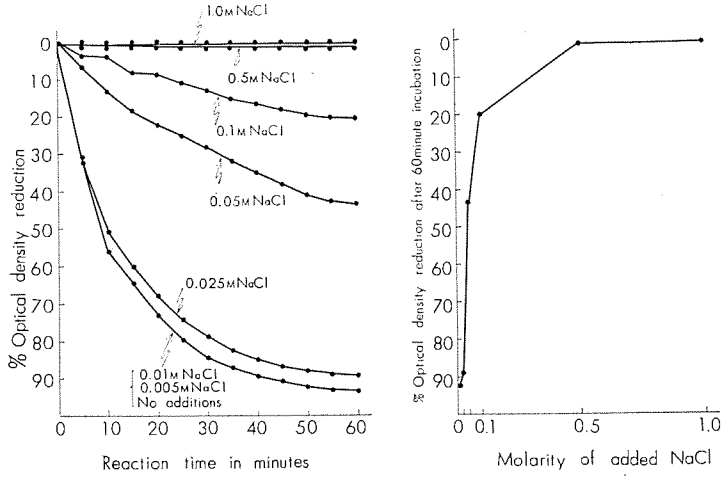


Fig. 8. Effect of Ionic Strength on the Activity of the Lytic Factor

NaCl was added to reaction mixtures containing 0.01 M Tris-HCl buffer (pH 7.2) at the concentrations indicated.

6) *Activity range of the lytic factor*

It has been demonstrated in a previous study (Kotani *et al.*, 1959b) that L_{11} bacterium produced a distinct, clear zone around its colonies on an agar plate containing either cell walls or cells of *Strept. pyogenes* (Group A), *C. diphtheriae* and BCG other than *Staph. aureus*. Specimens of the $ZnCl_2$-concentrate were therefore assayed for their lytic activity against cell wall suspensions of these organisms and of *M. lysodeikticus*. As shown in Fig. 9, while *M. lysodeikticus* cell walls were very susceptible to the lytic factor, the cell walls of all the other organisms tested were scarcely lysed.

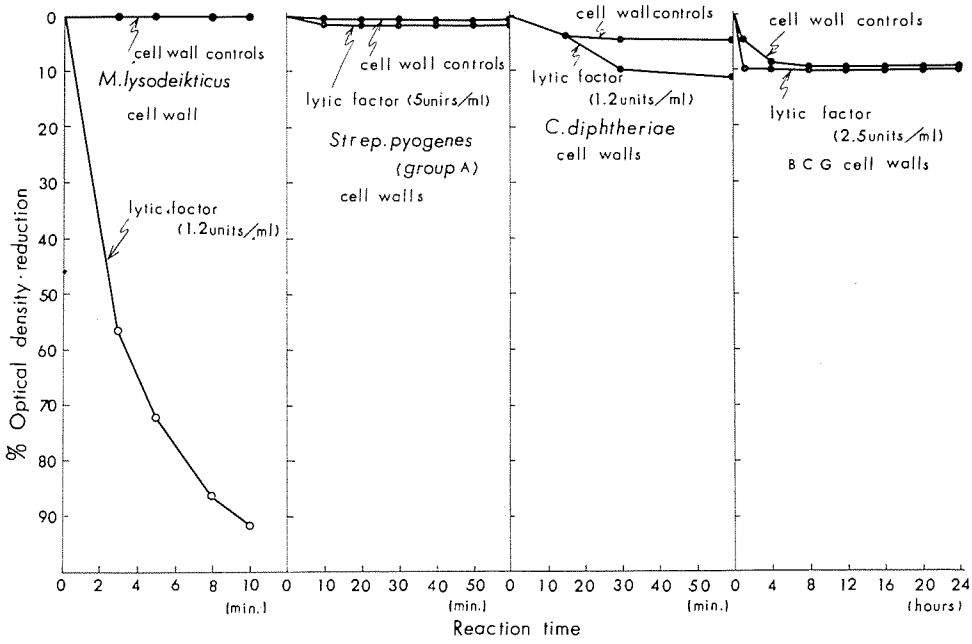


Fig. 9. Activity Range of the Lytic Factor

3. *Analysis of degradation products of Staph. aureus cell walls and their mucopeptide fraction on lysis with L_{11} enzyme*

Specimens of L_{11} enzyme purified by column chromatography were used in the following experiments.

1) *Propertis of Staph. aureus cell walls and a mucopeptide fraction derived from them*

The data on the chemical properties of the cell walls and cell wall mucopeptide fraction of *Staph. aureus* are summarized in Table 2. The findings are essentially

Table 2. Chemical Properties of Cell Walls and Cell Wall Mucopeptide of *Staph. aureus*

Materials	Nitrogen per cent	Phosphorus per cent	Hexosamine*1 per cent	Main component amino acids	Main component sugars
Cell walls	12.1	1.76	25.5	alanine glutamic acid lysine glycine	glucosamine (muramic acid)*2
Cell wall mucopeptide	14.6	0.38	15.6	alanine glutamic acid lysine glycine	glucosamine (muramic acid)*2

*1 Expressed as glucosamine-HCl. Determinations were made with the specimens hydrolyzed in 4 N HCl for 4 hours at 100°C.

*2 No authentic specimen of muramic acid was available for reference.

the same as those reported by other workers (Strominger, Park and Thompson, 1959; Mandelstam and Strominger, 1961; Morse, 1962).

Fig. 10 illustrates the electron microscopic appearance of *Staph. aureus* cell walls (A) and that of a cell wall lysate obtained under the action of the lytic enzyme (B). It can be seen from their morphological appearance that the cell walls of *Staph. aureus* completely lost their original shape.

As regards the susceptibility of *Staph. aureus* cell walls to enzymes other than L_{11} enzyme, it was demonstrated (Fig. 11) that they did not exhibit any significant optical density reduction on incubation for 60 minutes at 37°C with crystalline egg white lysozyme (prepared by the method of Alderton, 1946; 1 mg/ml), Nagarse (a crystalline protease of *Bacillus subtilis var. Biotecus A*, from Teikoku Chemical Industry, Tokyo; 1250 units/ml) or Pronase (a protease of *Streptomyces griseus*, from Kaken Chemicals Co., Tokyo; 2515 units/ml). The figures cited above indicate the final concentration of the enzyme preparations in the reaction mixture.

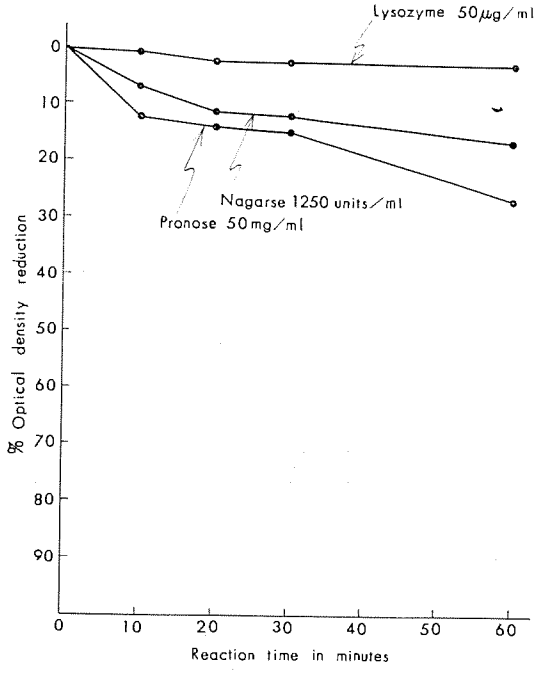


Fig. 11. Susceptibility of *Staph. aureus* Cell Walls to Egg White Lysozyme and Bacterial Proteases

2) *Fractionation of cell wall lysate*

Three hundred mg of cell wall preparation were suspended in 35 ml of 0.02 M Tris-HCl buffer (pH 7.2) containing 325 units of L_{11} enzyme and 0.1 per cent NaN_3. A 0.4 ml aliquot of the reaction mixture was withdrawn prior to incubation

and diluted 10-fold with 0.02 M Tris-HCl buffer (pH 7.2) containing 0.1 per cent NaN_3. Both the pilot and main reaction mixtures were incubated at 37°C for 48 hours. The reduction in optical density in the pilot tube was determined at intervals and was found to be 79.5 and 95.5 per cent after incubation for 60 and 120 minutes, respectively. After incubation for 48 hours, the lysates from both the main and pilot reaction mixtures were combined and dialyzed in seamless cellulose tubing, against 3 portions of 150 ml each of distilled water for 24 hours in the cold room. The dialyzate thus obtained was lyophilized and the material was kept *in vacuo* over P_2O_5. The non-dialyzable portion of the lysates was further dialyzed against a large quantity of distilled water for another 24 hours. The 42 ml of fluid in the cellulose tubing was mixed with 10.5 ml of 50 per cent (w/w) trichloroacetic acid solution to remove enzyme protein. The supernatant fluid, separated from a small amount of precipitate by centrifugation at 13,000 *g* for 10 minutes, was then mixed with 5 volumes of ethyl alcohol. After overnight storage in the cold room, a precipitate was collected by centrifugation, washed twice with 50 ml portions of ethyl alcohol and ether and dried *in vacuo* (yield 90.9 mg). The supernatant was put into cellulose tubing and concentrated by pervaporation overnight in the cold room. The concentrate was dialyzed against distilled water and lyophilized (53.0mg). A similar fractionation experiment was performed with lysates derived from a reaction mixture which contained 190 mg of a cell wall preparation and 450 units of L_{11} enzyme and was incubated for 24 hours.

Table 3. Relative Proportions of Dialyzable and Non-Dialyzable Fraction (Alcohol-Soluble and Alcohol-Precipitable) in Cell Wall Lysate

Experiment	Cell walls lysed	Non-dialyzable fraction		Dialyzable fraction*2
No	mg	Alcohol-soluble fraction mg (per cent)	Alcohol-precipitable fraction mg (per cent)	per cent
1	300	53.0 (17.7)*1	90.9 (30.3)	52.0
2	190	33.9 (17.8)	75.0 (39.5)	42.7

*1 Expressed as percentage of cell walls examined.

*2 Calculated by substracting the weight of the non-dialyzable fraction from that of the original cell walls.

The relative proportions of the three fractions isolated from the cell wall lysates in these experiments are presented in Table 3. The table shows that approximately half the wall components were converted to dialyzable compounds under the action of L_{11} enzyme and that approximately two thirds of the non-dialyzable portion of the cell wall lysates was precipitated with ethyl alcohol. The chemical properties of these fractions will be described later.

3) *Quantitative analysis of components released from cell walls and cell wall mucopeptide by the action of the lytic enzyme*

A. *Lysis of cell wall*

Sixty mg of cell wall preparation were suspended in 12 ml of 0.01 M Tris-HCl buffer (pH 7.2) containing 60 units of L_{11} enzyme and 0.1 per cent NaN_3. A 0.4 ml aliquot of the suspension was withdrawn prior to incubation, diluted 10-fold with 0.01 M buffer containing 0.1 per cent NaN_3 and the reduction in its optical density was measured. The reaction mixtures were incubated at 37°C. Aliquots of 0.9 ml of the reaction mixture were withdrawn at intervals during the 8 hour incubation period After withdrawal they were immediately mixed with 0.1 ml of 5 M NaCl to stop enzyme action and centrifuged at 13,000 *g* for 30 minutes to remove insoluble materials, mainly unlysed cell walls and their fragments. The supernatants thus obtained were assayed for the content of ninhydrin-positive compounds and hexosamines.

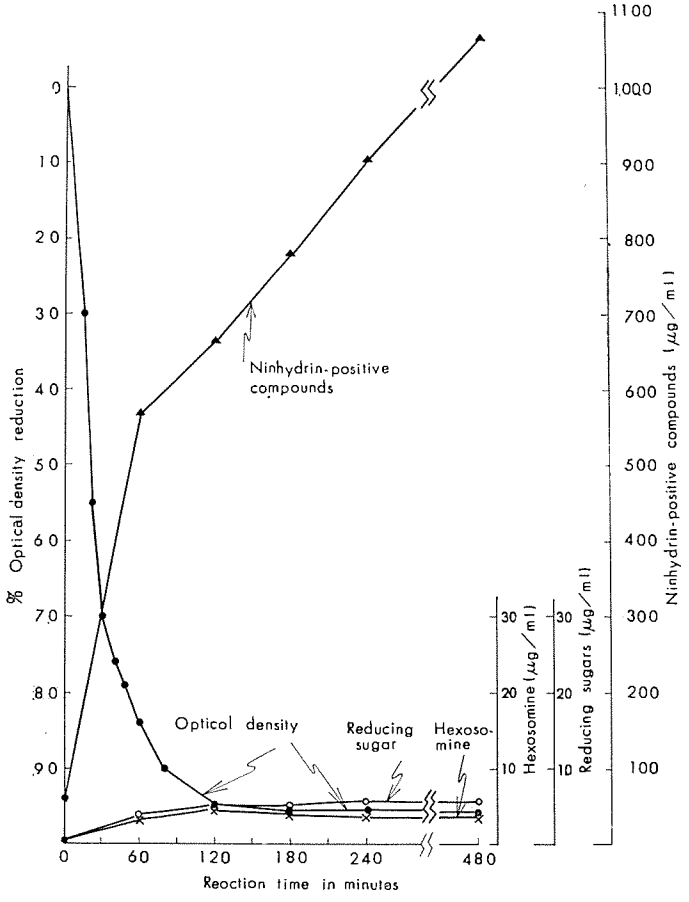


Fig. 12. Lysis of *Staph. aureus* Cell Walls by L_{11} Enzyme and Liberation of Ninhydrin-Positive Compounds

Fig. 12 indicates that the lysis of *Staph. aureus* cell walls by L_{11} enzyme was accompanied by a marked liberation of ninhydrin-positive compounds, but not by any significant release of compounds containing detectable hexosamine.

B. *Lysis of cell wall mucopeptide*

A mucopeptide fraction isolated from *Staph. aureus* cell walls was used as substrate for both L_{11} enzyme and egg white lysozyme. A specimen of 80.5 mg of the mucopeptide fraction was suspended in 25 ml of 0.05 M Tris-HCl buffer (pH 7.2) containing 225 units of L_{11} enzyme and 0.1 per cent NaN_3 (reaction mixture 1). Reaction mixture 2 contained 82.5 mg of the mucopeptide fraction and 2 mg of crystalline egg white lysozyme in a total volume of 25 ml. Before incubation, 0.5 ml aliquots were withdrawn from each of the two reaction mixtures for optical density determination, as in the previous experiments. After 4, 8 and 48 hours incubation at 37°C, aliquots of 5, 5 and 14 ml respectively were withdrawn from each of the reaction mixtures and centrifuged at 13,000 *g* for 20 minutes. The supernatant fluids thus obtained were analyzed for compounds reacting with ninhydrin and compounds detectable as reducing sugars, hexosamines or N-acetyl-

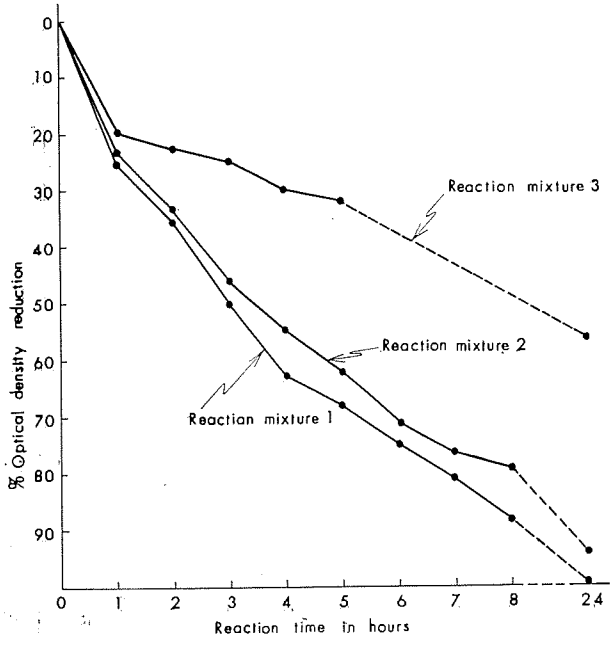


Fig. 13. Lysis of *Staph. aureus* Cell Wall Mucopeptide by L_{11} Enzyme or/ and Egg White Lysozyme

Reaction mixture 1 contained 2.8 units of L_{11} enzyme per mg mucopeptide; mixture 2, 24 μg of egg white lysozyme per mg mucopeptide; and mixture 3, 2.6 units of L_{11} enzyme per residues derived from 1 mg of mucopeptide after exposure to egg white lysozyme for 48 hours (se reaction mixture 2).

hexosamines. Aliquots of 8 ml of the supernatants from the specimens withdrawn from 48 hour lysates were dialyzed twice against 50 ml of distilled water. The dialyzable fractions were lyophilized and analyzed by paper chromatography.

Fig. 13 illustrates changes in the optical density of reaction mixtures 1 and 2, indicating that the cell wall mucopeptide of *Staph. aureus*, unlike the original cell wall, was susceptible to both egg white lysozyme and L_{11} enzyme. Similar observations on the susceptibility of the cell wall mucopeptide of *Staph. aureus* towards egg white lysozyme have recently been reported by Mandelstam and Strominger (1961) and Morse (1962).

The results of chemical analysis are given in Table 4. It can be seen from this table that the chemical process inducing lysozyme lysis of cell wall mucopeptide are entirely different from that causing its lysis by L_{11} enzyme. Lysis of the mucopeptide fraction by L_{11} enzyme was accompanied by the marked release of materials which reacted with ninhydrin but showed no reducing activity, wheras lysis of the same fraction under the action of egg white lysozyme resulted in the liberation of reducing compounds giving a positive hexosamine and negative ninhydrin reaction.

Table 4. Quantitative Chemical Analysis of the Lysates of Cell Wall Mucopeptide Produced by the Action of L_{11} Enzyme and Lysozyme

Reaction mixture			Period of incubation	Optical density reduction	Ninhydrin positive *1 compounds	Reducing *2 sugars	Hexosamine *3	N-acetylhexosamine*4 Heated for	
No.	Composition	Volume ml	hours	per cent	μg/ml	μg/ml	μg/ml	3 mins μg/ml	60 mins μg/ml
1	80.5 mg mucopeptide and 225 units of L_{11} emzyme	25	4	63	510	0	2.5	0	0
			8	81	590	0	3.0	0	0
			48	95	840	0	3.5	0	0
2	82.5 mg mucopeptide and 2 mg lysozyme	25	4	54	27	7.7	8.7	2.3	11.4
			8	76	33	15.5	13.0	5.0	26.7
			48	94	94	37.7	25.5	5.0	28.5
3	Residue derived from 48 hour lysate (12.8 ml)of reaction mixture 2 and 108 units of L_{11} enzyme	12	72	54	310	22.4	17.4	0	3.5

All determinations were performed on unhydrolyzed specimens.

*1 Expressed as L-leucine.

*2 Expressed as glucose.

*3 Expressed as glucosamine-HCl.

*4 See Text.

Reaction mixture 3 in Fig. 13 and Table 4 was set up by suspending the insoluble residue obtained by centrifugation of 12.8 ml of 48 hour lysate from reaction mixture 2 in 12 ml of 0.05 M Tris-HCl buffer (pH 7.2) containing 108 lytic units of L_{11} enzyme and 0.1 per cent NaN_3. The optical density determination and

chemical analysis of this reaction mixture were performed in the same manner as for reaction mixtures 1 and 2. It should be noted that the insoluble residue remaining after treatment of cell wall mucopeptide with lysozyme was lyzed on addition of L_{11} enzyme. This lysis was accompanied by marked release of ninhydrin-positive compounds.

4) *Analysis by paper chromatography of the dialyzable fraction*

Lyophilized specimens of the dialyzable fraction from the mucopeptide lysates (see section 3-3)-B), were dissolved in an appropriate amount of distilled water for paper chromatographic analysis.

Developed by the ascending procedure using *n*-butanol-acetic acid-water (3 : 1 : 1, v/v) as solvent

Fig. 14. Paper Chromatogram of Dialyzable Fraction of *Staph. aureus* Cell Wall Mucopeptide Produced by L_{11} Enzyme

A suitable amount of the specimens was applied to a strip of No. 51 filter paper and the strip was developed by the ascending procedure using *n*-butanol-acetic acid-water as solvent. As shown in Fig. 14, when sprayed with ninhydrin reagent, the chromatogram showed 4 distinct spots.

To isolate the material from each spot for further analysis, the sample was streaked as narrow band, 38 cm long, on a 40 cm × 40 cm sheet of No. 51 filter paper and the sheet was developed at 28°C for 88 hours by descending chromatography using *n*-butanol-acetic acid-water as solvent. Three strips, 1.5 cm in width, were cut off from the sides and center of the paper after development and sprayed to localize ninhydrin positive spots. Using these as guides, the following 4 bands were cut off from the unsprayed paper: (1) 6.8 - 8.4 cm band, (2) 9.0 - 11.2 cm band, (3) 16.5 - 17.7 cm band and (4) 24.2 - 27.7 cm band (the figures cited above indicate the distance of the bands from the origin). These bands were extracted by immersion in 15 ml of distilled water. The extracts were filtered through sintered glass filters (3G4) and the filtrates were dried over P_2O_5 *in vacuo*. The

Table 5. Analysis by Paper Chromatography of 4 Main Ninhydrin-Positive Compounds Hydrolyzed and Isolated from a Cell Wall Mucopeptide Lysate

Materials	Rf value		
	phenol-water-ammonia (80:40:0.3, v/v)	*n*-butanol-pyridine-water (6:4:3, v/v)	*n*-butanol-acetic acid-water (3:1:1, v/v)
DL-alanine	0.64	0.22	0.44
Spot 1 A	0.62	0.20	0.45
2 A	0.61	0.21	0.44
3 A	0.64	—	—
4 A	—	—	—
L-glutamic acid	0.32	0.07	0.39
Spot 1 B	0.30	0.09	0.40
2 B	0.30	0.09	0.38
3 B	0.27	—	0.38 tr
4 B	—	—	—
Glycine	0.47	0.15	0.34
Spot 1 C	0.43	0.14	0.32
2 C	0.43	0.14	0.32
3 C	0.47	0.14	0.31
4 C	0.46	0.13	0.32
L-Lysine	0.81	0.034	0.24
Spot 1 D	0.79	0.028	0.20
2 D	0.79	0.028	0.20
3 D	—	—	0.18
4 D	—	—	0.18 tr
D-glucosamine	0.65	0.32	0.32
Spot 1	—	—	—
2	—	—	—
3	—	—	—
4	—	—	—

Paper chromatography was performed by ascending procedure on specimens hydrolyzed in 6 N HCl for 16 hours at 100°C.

dried materials were then dissolved in 3 ml portions of 6 N HCl and hydrolyzed at 100°C for 16 hours. The hydrolyzates were kept over P_2O_5 and NaOH *in vacuo* to remove HCl and the dried materials were redissolved in an appropriate amount of distilled water. The materials thus obtained were chromatographed by both ascending and descending procedures using phenol-water-ammonia, *n*-butanol-acetic acid-water, and *n*-butanol-pyridine-water as developing solvents. The results obtained by the ascending procedure are summarized in Table 5. From these results and other available data, it is concluded that the 4 compounds corresponding to ninhydrin-positive spots had the following amino acid compositions. The compounds corresponding to spots 1 and 2 were peptides containing alanine, glutamic acid, lysine and glycine and the compound detectable as spot 4 only contained glycine. The compound localized in spot 3 seems to have an intermediate amino aid composition (Table 6).

Table 6. Amino Acid Composition of 4 Main Ninhydrin-Positive Compounds Isolated from Cell Wall Mucopeptide Lysate Produced by L_{11} Enzyme

Compound	Amino acids detected				
	alanine	glutamic acid	lysine	glycine	glucosamine
Spot 1	+++	+++	+++	+++	—
2	+++	+++	+++	+++	—
3	tr	tr	tr	+++	—
4	—	—	—	+++	—

Only one spot was detected by the $AgNO_3$ method of Trevelyan for reducing substances, on chromatograms developed in any of the above solvents. However, this compound did not react with either aniline hydrogen phthalate or the reagent used in Elson and Morgan's method. It was further demonstrated that a hydrolyzate of the material extracted from this spot was localized at the same position as the original material. The nature of this compound is still unknown.*

5) *Analysis of the non-dialyzable components*

Specimens of both alcohol-soluble and alcohol-precipitable fractions obtained from the non-dialyzable portion of the cell wall lysate (see section 3-2)) were used in the following study.

Samples of 2 mg each of the fractions were added to 5 ml portions of 4 N HCl and the mixtures were heated at 100°C for 4 hours. The hydrolyzates of the specimen were kept over P_2O_5 and NaOH. The dried materials were then dissolved in 2 ml portions of 2 N HCl and applied to a 6 mm × 55 mm column prepared

* Since this paper was submitted for publication, the authors noticed that Ghuysen and Salton (1960) had described a similar spot which reacted with the silver nitrate reagent. They stated that it originated from the dialysis tubing and appeared to be glycerol or a related substance.

from a slurry of 0.25 g each of Norit Extra and Celite No. 535. Elution was performed successively with 15 ml each of distilled water and 5 per cent (v/v) ethyl alcohol in distilled water. The volume of the effluents was adjusted to 20 ml. A 2 ml aliquot of each effluent was dried over P_2O_5 and NaOH. The materials, redissolved in appropriate amount of distilled water, were used for the differential determination of glucosamine and muramic acid. A specimen of the cell wall preparation was treated in the same manner. The content of ninhydrin-positive compounds was determined on the specimens hydrolyzed in 4N HCl at 100°C for 16 hours.

Table 7. Chemical Analysis of Alcohol-Soluble and Alcohol-Precipitable Fractions Isolated from Non-Dialyzable Portion of Cell Wall Lysate Produced by L_{11} Enzyme

Material	Hexosamine		Ninhydrin-positive compounds	Phosphorus
	glucosamine per cent	muramic acid per cent	per cent	per cent
Cell walls	25.0	12.0	86.8	1.76
Non-dialyzable portion of cell wall lysate				
Alcohol-soluble fraction	37.5	21.5	48.0	0.19
Alcohol-precipitable fraction	34.5	12.5	36.0	5.68

All figures are percentages of values for total material examined.

The results are given in Table 7. It can be seen that the hexosamine content of both the alcohol-soluble and alcohol-precipitable fractions was very high and they contained nearly 60 and 50 per cent hexosamines (glucosamine and muramic acid), respectively. The hexosamine content in the dialyzable fraction (not shown in the table), on the other hand, was only 0.5 per cent (the differential determination of glucosamine and muramic acid was not performed with this fraction). The table also shows that the alcohol-precipitable fraction contained almost 30-times as much phosphorus and about half as much muramic acid as the alcohol-soluble fraction. There was no significant difference between their contents of ninhydrin positive compounds. Paper chromatography of hydrolyzed specimens of both the fractions indicated the presence of alanine as the main constituent amino acid.

DISCUSSION

Analysis of the products split off and the chemical process involved during lysis of bacterial cell walls by appropriate lytic enzymes should provide one of the most effective means of elucidating the chemical structure of the cell walls. A considerable number of investigations have been made along these lines. Representative investigations in this field are those of Ghuysen, Salton, Perkins and

others on *M. lysodeikticus* cell walls. These workers used egg white lysozyme and/or F_1 and F_2B enzymes of *Streptomyces albus G* (Ghuysen and Salton, 1960; Ghuysen, 1960; Salton and Ghuysen, 1960; Perkins, 1960a, b). Studies have also been made on lysozyme lysates of *Escherichia coli* (Primosigh *et al.*, 1961) and cell wall lysates produced by *Streptomyces albus* enzyme of *Strept. pyogenes*, Group A (McCarty, 1952b).

The high specific activity of the enzyme preparation isolated by chromatography on hydroxylapatite, ranging from 2,000 to 4,000 units/mg protein N, suggests that the preparations obtained were fairly pure. It is worth mentioning that the specific lytic activity of crystalline preparations of egg white lysozyme against *M. lysodeikticus* cell walls has been shown to be in the order of 3,000 units/mg protein N, if one lytic unit is defined as in the present study described above.

The course of lysis and the products of lysis of *Staph. aureus* cell walls have been studied both with cell walls themselves and the cell wall mucopeptide derived from them, since mucopeptide seems to be the basal structure of the cell walls of a variety of bacteria (Armstrong *et al.*, 1958; Park and Hancock, 1960; Krause and McCarty, 1961; Rogers, 1962). Moreover the cell wall mucopeptide of some lysozyme-resistant bacteria, unlike the original cell walls, has recently been demonstrated to be susceptible to egg white lysozyme (Mandelstam and Strominger, 1961, and Morse, 1962, for *Staph. aureus;* Krause and McCarty, 1961, for *Strept. pyogenes*, Group A; Mandelstam, 1961, for *Esch. coli*).

In confirmation of the studies of Mandelstam and Strominger and Morse, a mucopeptide fraction obtained by extraction of *Staph. aureus* cell walls (Newman 1 strain) with 5 per cent trichloroacetic acid was proved to be susceptible to egg white lysozyme. The chemical process inducing lysozyme lysis of the cell wall mucopeptide, however, has been demonstrated to be entirely different from that causing its lysis by L_{11} enzyme: while lysozyme lysis was accompanied by release of reducing compounds giving a positive hexosamine reaction in the unhydrolyzed state, the lysis of the mucopeptide by L_{11} enzyme resulted in liberation of ninhydrin-positive compounds with free amino groups, but did not release any significant amount of reducing substances. Moreover, paper chromatography of dialyzable components of the lysates of the mucopeptide produced by L_{11} enzyme demonstrated peptides of at least two sorts, one of which contained alanine, glutamic acid, lysine and glycine and the other which contained only glycine.

Recently, Mandelstam and Strominger (1961) proposed two possible chemical structures for *Staph. aureus* cell walls, one of which is presented in Fig. 15. On the basis of this proposed structure, the findings reported here would be interpreted as indicating that L_{11} enzyme primarily attacks -CO-NH- bonds between the lactyl group of muramic acid and the amino group of alanine and those between lysine or glutamic acid and glycine. Further studies are now in progress on the action of L_{11} enzyme upon so-called Park's nucleotides which are believed to be precursors of *Staph. aureus* cell walls and which have been shown to accumulate during growth

of this organism in the presence of penicillin (Strominger, 1962).

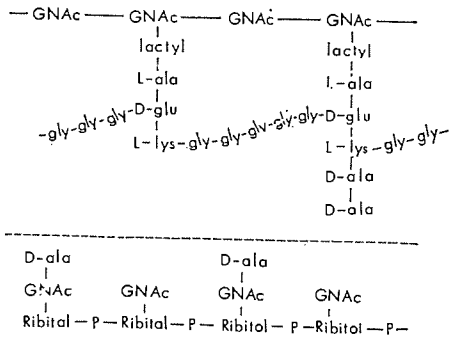


Fig. 15. The Chemical Structure of *Staph. aureus* Cell Walls Proposed by Mandelstam and Strominger (1961)

Although analysis of the non-dialyzable fraction of cell wall lysates by L_{11} enzyme are very limited at present and further work is necessary, available chemical data and serological findings to be reported elsewhere suggest that the fraction is composed both of a polymer consisting of glucosamine and muramic acid and of components containing ribitol teichoic acid as the main constituent.

There have been three studies on the lytic enzymes active against *Staph. aureus* cell walls (two of these appeared after the present work had been started): a study of Ghuysen (1957) on the F_1 enzyme produced by *Streptomyces albus G*, prelimary reports by Schindler and Schuhard (1961, 1962) on an enzyme produced by a Gram-positive *Micrococcus* (K-6-WI) and by Hash (1962) on a staphylolytic factor produced by a fungus, *Chalaropsis sp*. Although F_1 enzyme has been reported to act as a peptidase on the cell walls of *Bacillus megaterium* and *Staph. aureus*, this enzyme preparation was shown to exert β-(1-4) N-acetylhexosaminidase activity on *M. lysodeikticus* cell walls (Ghuysen and Salton, 1960; Ghuysen, 1960; Salton and Ghuysen, 1961). Although there seem to be some similarities between Ghuysen's F_1 enzyme and L_{11} enzyme reported in this paper, further investigations are required on their relationship. The enzyme produced by *Chalaropsis sp.*, on the other hand, is distinctly different from L_{11} enzyme, since the former enzyme has been reported to act as an N-acetylhexosaminidase. No information is yet available on the nature of the substrate(s) of the lytic factor reported by Schindler and Schuhard.

REFERENCES

Alderton, G. (1946). Direct crystalization of lysozyme from egg white and some crystalline salts of lysozyme. *J. Biol. Chem.* **164,** 1-5.

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. and Greenberg, G.R. (1958). Isolation and structure of ribitol phosphate derivatives (Teichoic acids) from bacterial cell wall. *J. Chem. Soc.* **88,** 4344-4354.

Fister, H.J. (1950). *Manual of standardized procedures for spectrophotometric chemistry.* Method P-38a.1, Standard Scientific Supply Cooperation, New York.

Ghuysen, J. M. (1957). Activitès bactérioloytiques de l'actinomycetine de *Streptomyces albus. Arch. Intern. Phys. Biochem.* **65,** 173-306.

Ghuysen, J. M. and Salton, M. R. J. (1960). Acetylhexosamine compounds enzymatically released from *Micrococcus lysodeikticus* cell walls. I. Isolation and composition of acetylhexosamine and acetylhexosamine-peptide complexes. *Biochim. Biophys. Acta* **40,** 462-472.

Ghuysen, J. M. (1960). Acetylhexosamine compounds enzymatically released from *Micrococcus lysodeikticus.* II. Enzymic sensitivity of purified acetylhexosamine and acetylhexosamine-peptide complexes. *Biochim. Biophys. Acta* **40,** 473-480.

Gomori, G. (1948). *Proc. Soc. Exptl. Med.* **68,** 354 (cited in *Methods in Enzymology* (edited by Colowick, S. P. and Kaplan, N. O. (1955), Vol. 1, p. 143. Academic Press, New York)).

Hash, J. H. (1962). Formation of *Staphylococcus aureus* protoplasts by enzymes from *Chalaropsis sp. Fed. Proc.* **21,** 248F.

Kotani, S., Kitaura, T., Hirano, T. and Tanaka, A. (1959a). Isolation and chemical composition of the cell walls of BCG. *Biken's J.* **2,** 129-141.

Kotani, S., Hirano, T., Kitaura, T., Kato, K. and Matsubara, T. (1959b). Studies on the isolation of bacteria capable of lysing lysozyme-resistant, pathogenic bacteria. *Biken's J.* **2,** 143-150.

Kotani, S., Kato, K., Matsubara, T., Hirano, T. and Higashigawa, T. (1959c). *Staphylolytic* activity of a culture filtrate of a *Flavobacterium species,* isolated from soil. *Biken's J.* **2,** 211-213.

Kotani, S., Harada, K., Kitaura, T., Hashimoto, S, and Chimori, M. (1962). Enzymatic lysis of BCG cell walls. *Biken J.* **5,** 117-119.

Krause, R. M. and McCarty, M. (1961). Studies on the chemical structure of the *streptococcal* cell wall. I. The identification of a mucopeptide in the cell walls of group A and A variant *streptococci. J. Exptl. Med.* **114,** 127-140.

Krause, R. M. and McCarty, M. (1962). Studies on the chemical structure of the *streptococcal* cell wall. II. The composition of group C cell walls and chemical basis for serological specificity of the carbohydrate moiety. *J. Exptl. Med.* **115,** 49-62.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Radall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Mandelstam, J. (1961). Isolation of lysozyme soluble mucopeptides from cell walls of *Escherichia coli. Nature* **189,** 855-856.

Mandelstam, M. H. and Strominger, J. L. (1961). On the structure of the cell walls of *Staphylococcus aureus* (Copenhagen). *Biochem. Biophys. Res. Commun.* **5,** 466-471.

McCarty, M. (1952a). The lysis of group A hemolytic *streptococci* by extracellular enzymes of *Streptomyces albus.* I. Production and fractionation of the lytic enzymes. *J. Exptl. Med.* **96,** 555-568.

McCarty, M. (1952b). The lysis of group A hemolytic *streptococci* by extracellular enzymes of *Streptomyces albus.* II. Nature of the cellular substrate attacked by the lytic enzymes. *J. Exptl. Med.* **96,** 569-580.

Moore, S. and Stein, W.H. (1948). Photoelectric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* **176,** 367-388

Mori, Y., Kato, K., Matsubara, T. and Kotani, S. (1960). Lysis of the cell walls of *Corynebacterium diphtheriae* by extracellular enzymes of *Streptomyces sp.* 1. Production and concentration of the lytic factor. *Biken's J.* **3,** 139-149.

Mori, Y. and Kotani, S. (1962). Lysis of the cell walls of *Corynebacterium diphtheriae* by extracellular enzymes of *Streptomyces sp.* 2. Production of "protoplasts" by treatment with a purified cell wall lytic enzyme. *Biken J.* **5,** 55-66.

Morse, I. (1962). Studies on the chemistry and immunochemistry of cell walls of *Staphylococcus aureus. J. Exptl. Med.* **116,** 229-245.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153,** 375-380.

Neuhaus, O. W. and Letzring (1957). Determination of hexosamines in conjunction with electrophoresis on starch. *Analyt. Chem.* **29,** 1230-1233.

Nomura, M., Hosoda, J. and Nishimura, S. (1958). Enzyme formation in lysozyme lysate of *Bacillus subtilis. Biochim. Biophys. Acta* **29,** 161-167.

Park, J. T. and Hancock, R. (1960). A fractionation procedure for studies of the synthesis of cell wall mucopeptide and of other polymers in cells of *Staphylococcus aureus. J. Gen. Microbiol.* **22,** 249-258.

Partridge, S. M. and Westall, R. G. (1948). Filter paper partition chromatography of sugars. I. General description and application to the quantitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42,** 238-250.

Partridge, S. M. (1949). Nature **164**, 443 (cited from Block, R. J., Durrum, E. L. and Zweig, G. (1955). *A manual of paper chromatography and paper electrophoresis*, p.133. Academic Press, New York).

Perkins, H.R. (1960a). The structure of a disaccharide liberated by lysozyme from the cell walls of *Micrococcus lysodeikticus. Biochem. J.* **74,** 182-186.

Perkins, H. R. (1960b). Substances reacting as hexosamine and as N-acetylhexosamine liberated from bacterial cell walls by lysozyme. *Biochem. J.* **74,** 186-192.

Perkins, H. R. and Rogers, H. J. (1959). The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus. Biochem. J.* **72,** 647-654.

Primosigh, J., Pelzer, H., Maass, D, and Weidel, W. (1961). Chemical characterization of mucopeptide released from the *E. coli* B cell wall by enzymic action. *Biochim. Biophys. Acta* **46,** 68-80.

Reissig, J. L., Strominger, J. L. and Leloir, L. F. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.* **217,** 959-966.

Rogers, H. J. (1962). The surface structures of bacteria. *Biochem. J.* **83,** 18-20p.

Salton, M. R. J. (1955). Isolation of *Streptomyces spp.* capable of decomposing preparations of cell walls from various microorganism and a comparison of their lytic activities with those of certain *Actinomycetes* and *Mycobacteria. J. Gen. Microbiol.* **12,** 25-30.

Salton, M. R. J. and Ghuysen, J. M. (1960). Acetylhexosamine compounds enzymatically released from *Micrococcus lysodeikticus* cell walls. III. The structure of di-and tetra-saccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme. *Biochim. Biophys. Acta* **45,** 355-363.

Schindler, C. A. and Schuhardt, V. T. (1960). *Staphylolytic* factor(s) produced by a gram positive coccus. *Bacteriol. Proc.* p.82.

Schindler, C.A. and Schuhardt, V. T. (1961). *Staphylolytic* enzyme from a *micrococcus* (K-6-WI). *Bacteriol. Proc.* p.126.

Strominger, J. L. (1962). Biosynthesis of bacterial cell wall. *Fed. Proc.* **20,** 134-143.

Strominger, J. L., Park, J. T. and Thompson, R. E. (1959). Composition of the cell wall of *Staphylococcus aureus*: Its relation to the mechanism of action of penicillin. *J. Biol. Chem.* **234,** 3263-3268.

Tiselius, A., Hjertén, S. and Levin, Ö. (1956). Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* **65,** 132-155.

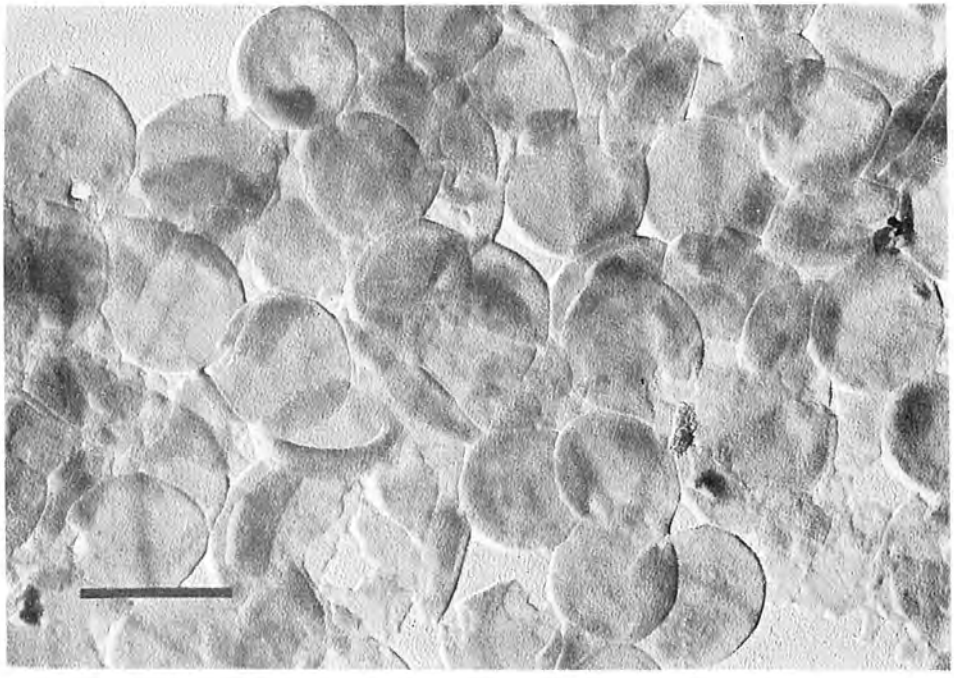
Trevelyan, W. E., Procter, D. P. and Harrison, J. S. (1950). Detection of sugars on paper chromatograms. *Nature* **166,** 444-445.

Yokoi, H. and Akasi, S. (1955). An improved Kjeldahl-Nessler method. *Nagoya Med. J.* **3,** 91-104.

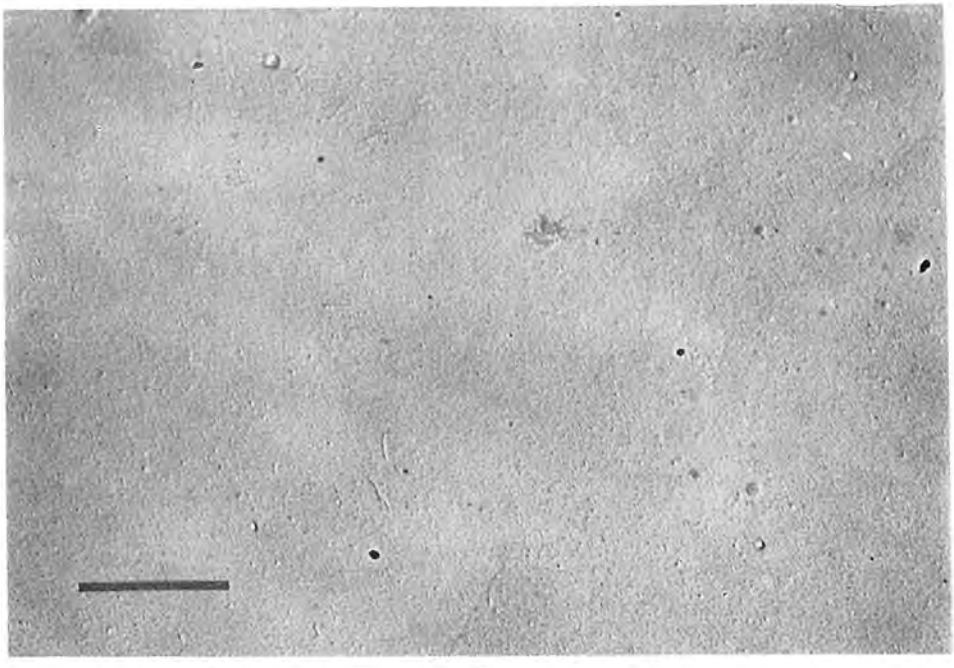
EXPLANATION OF PHOTOGRAPHS

Fig. 10. Electron Microscopic Appearance of *Staph. aureus* Cell Walls and Lysate of Cell Walls by L_{11} Enzyme

(A): *Staph. aureus* cell walls, (B): Lysate of cell walls incubated with lytic enzyme (1.5 units/ml final concentration) for 2 hours at 37° C. The specimens were mounted on collodion films and shadowed with chromium. Scale: 1 μ.



(A)



(B)

Fig. 10.