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Lysis of the Cell Walls of *Corynebacterium diphtheriae* by Extracellular Enzymes of *Streptomyces sp.* 1. Production and Concentration of the Lytic Factor

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SUMMARY

An investigation has been made to isolate and concentrate a lytic enzyme active against both the cell wall preparations and intact cells of *Corynebacterium diphtheriae* from culture filtrates of *Streptomyces sp.* (named L_3 bacterium) isolated from soil. The filtrate of a 7 day old culture of L_3 bacterium grown on modified McCarty's medium exhibits a marked lytic activity against *Corynebacterium diphtheriae* and the active factor can be concentrated about 2.5 times per mg protein N by 0.6 saturation of the culture filtrate with solid $(NH_4)_2SO_4$. A 25-fold by volume concentrate of the filtrate thus obtained induces 70 and 90 per cent reduction in optical density of cell wall and intact cell suspensions of *Corynebacterium diphtheriae*, respectively. The same concentrate exhibits a very marked lytic action against intact cells of *Bacillus megaterium*, but does not attack *Micrococcus lysodeikticus, Sarcina lutea, Sarcina valiabilis, Staphylococcus aureus, Streptococcus pyogenes* (group A) and BCG.

The active enzyme is heat-labile, and is almost completely inactivated by heating it at 60°C for 30 minutes. The lytic activity, however, is stable for at least a month when the preparation is stored in a refrigerator. The optimum pH for the lytic activity is between 7.5 and 8.5.

Data are also presented on the chemical nature of the cell walls of *Corynebacterium diphtheriae* used in this study.

INTRODUCTION

In recent years striking advances have been made in the field of bacterial anatomy and it has become possible to investigate various activities of microorganisms at a subcellular level. The rapid progress in this field is due largely to the utilization of the cell wall lytic activity of egg white lysozyme.

Unfortunately, however, most pathogenic microorganisms, especially Gram-positive ones, are resistant to egg white lysozyme and lysozyme is of little or no use for analysing the various properties peculiar to these resistant pathogens.

For the past years, studies have been made in the author's laboratory on cell wall lytic agents active against lysozyme-resistant, pathogenic microorganisms. Microorganisms capable of lysing the cell walls of these organisms were isolated from soil samples. Some of the results obtained have already been reported in previous papers (Kotani *et al.*, 1959a, b). The present report describes the produc-

* A preliminary report of part of this work was presented at the 7th Symposium on Bacterial Toxins, at Hakone, Kanagawa, in May 1960.

tion of the lytic enzyme active against *Corynebacterium diphtheriae* by *Streptomyces sp.* isolated from a soil sample.

MATERIALS AND METHODS

1. *Streptomyces sp. capable of lysing C. diphtheriae*

Streptomyces sp. (provisionally named L_3 bacterium) which produces a distinct zone of lysis during growth on an opaque medium containing *C. diphtheriae* cell walls or intact cells, was isolated from a soil sample collected at Kashiwara, Nara, by Salton's cell wall agar plate method (Salton, 1955a). Procedures used to isolate this organism were essentially the same as those described previously (Kotani *et al.*, 1959a), except that a *C. diphtheriae* cell wall agar plate was used as a primary isolation medium. The stock of L_3 bacterium was maintained by serial subculture on 0.1 per cent Bacto-peptone agar media (Bacto-peptone 1 g, K_2HPO_4 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, agar 20 g and distilled water 1,000 ml, pH 7.0).

2. *Culture media*

A variety of media were tested both for the growth of L_3 bacterium and for production of the lytic factor active against *C. diphtheriae.* The media used were as follows: 1) a modified McCarty's medium (McCarty, 1952a)-glucose 5 g, NaCl 5 g, K_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 1 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, $FeSO_4 \cdot 7H_2O$ 0.02 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g and a dialysate of Bacto-casamino acids, technical grade (5 g of casamino acids in Visking seamless cellulose tubing was dialyzed against one liter of distilled water at 4°C for 2 days and the outer fluid was used) 1,000 ml; 2) a modified Ghuysen's medium (Ghuysen, 1957)-polypeptone (Daigo Nutritional Chemical Co.) 10 g, $NaNO_3$ 2 g, KCl 0.5 g, K_2HPO_4 1 g, $Co(NO_3)_2 \cdot 7H_2O$ 0.03 g, $MgSO_4 \cdot 7H_2O$ 0.8 g, distilled water 1,000 ml; 3) a casamino acids-yeast extract medium-Bacto-casamino acids, technical grade, 1 g, Bacto-yeast extract 0.5 g, K_2HPO_4 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.25 g and distilled water 1,000 ml; and 4) a glucose-asparagine medium-glucose 10 g, asparagine 0.5 g, K_2HPO_4 0.5 g and distilled water 1,000 ml. All of these media, after being adjusted to pH 7.0, were sterilized by autoclaving (at 120°C for 10 minutes).

3. *Preparation of cell walls of C. diphtheriae, Staphylococcus aureus and BCG*

The cell materials and methods used for isolation of cell wall preparations have been fully described elsewhere (Kotani *et al.*, 1959a).

4. *Bacteria used to test the activity range of the lytic enzyme and bacterial growth conditions*

C. diphtheriae (strain Park-Williams No. 8, Toronto Harvard): 18-24 hour old cultures grown by shaking in a modified Taylor's medium.

Micrococcus lysodeikticus (strain No. 2665) and *Staph. aureus* (strain Newman 1): 18–24 hour old cultures grown by shaking in nutrient broth.

Streptococcus pyogenes, Lancefield group A (strain 089) : 18-24 hour old growth in Todd-Hewitt's medium.

Sarcina lutea (strain No. 3232), *Sarcina valiabilis* (strain No. 3062) and *Bacillus megaterium* (strain KM): 18-24 hour old cultures on nutrient agar medium.

BCG (strain Takeo): 10-12 day old cultures grown in modified Dubos' Tween 80-albumin medium (Schaefer *et al.*, 1949).

These organisms, after cultivation at 37°C, were collected either by centrifuging fluid cultures or by scraping the grown cells off agar slopes. They were washed with distilled water and finally suspended in distilled water at an appropriate optical density.

5. *Measurement of lytic activity*

The suspensions of intact cells or cell walls of test organisms were diluted with distilled water so that the diluted suspensions gave an optical density of approximately 0.3. To tubes (18±0.5 mm external diameter) containing 0.4 ml of the diluted intact cell or cell wall suspension were added 2.0 ml of the lytic enzyme solution appropriately diluted and 1.6 ml of 0.05 M Tris–(or 0.1 M phosphate–) buffer, pH 7.0. The tubes were incubated at 37°C in a water bath and the change in optical density of the reaction mixture was followed in a Hitachi photoelectric colorimeter, Type EPO–B, using a No. 55 filter.

One unit of the lytic activity against *C. diphtheriae* cell walls was provisionally defined as the amount of the lytic factor which caused 35 per cent (half maximal) decrease in the optical density of a reaction mixture after 60 minutes under the above conditions.

6. *Electron microscopic observation*

The techniques employed are essentially the same as those described previously (Kotani *et al.*, 1959c).

7. *Protein nitrogen determination*

To a sample of culture filtrate or of a fraction to be tested was added trichloroacetic acid to a final concentration of 0.4 M. The nitrogen content of both the untreated sample and the trichloroacetic acid soluble part was determined by the colorimetric method of Lowry et al. (1951). The difference in these two values was taken as the protein N content of of the sample.

8. *Analytical procedures*

A detailed description of the methods used in chemical analyses of cell wall preparations of *C. diphtheriae* was presented previously in a paper on the chemical properties of BCG cell walls (Kotani et al., 1959c).

RESULTS

1. *Cultural conditions for production of the lytic factor*

A variety of the culture media described under Materials have been tested for production of the active culture filtrate. Two hundred and fifty ml aliquots of these media were distributed as shallow layers in 500 ml Roux's bottles and each bottle was inoculated with 3 ml of the spore suspensions of L_3 bacterium. The suspension of spores was prepared by washing out with 6 ml of autoclaved distilled water one slant culture of L_3 bacterium which was grown on 0.1 per cent Bacto-peptone agar at 30°C for 7 days and then kept at room temperature for 3 to 5 weeks. The inoculated media were cultivated at 30°C for 2 weeks. Under these conditions the inoculated hydrophobic spores floated on the surface of the medium and germinated, leading to a continuous sheet of surface mycelial growth, which was heavily sporulated after 3 to 4 days cultivation. After various periods of cultivation, culture filtrate were obtained by filtration of the cultures through filter paper and were examined for their lytic activity against *C. diphtheriae* cell walls.

Fig. 1 shows the results of experiments on the *C. diphtheriae* cell wall lytic activity of the filtrates of L_3 bacterium grown on the four media for various periods. In this and the following Figures (except Figs. 2 and 4), per cent decrease

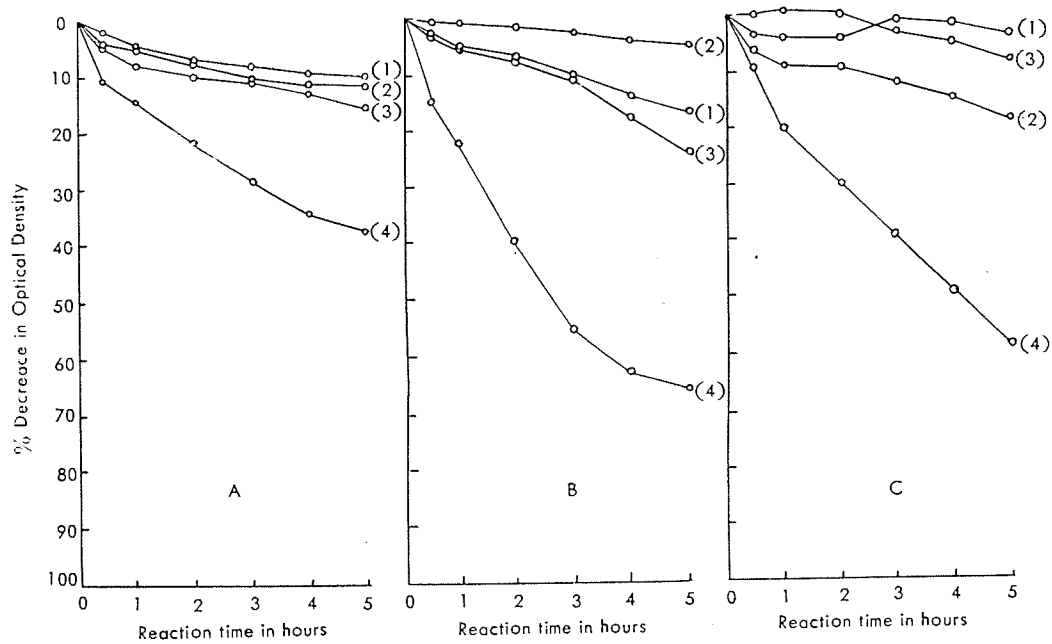

Fig. 1. Comparison of the lytic activity against C. *diphtheriae* cell walls of the culture filtrates of L_3 bacterium grown on various media for (A) 4 days, (B) 7days ond (C) 13 days. (1) Glucose-asparagine medium, (2) Ghuysen's medium, (3) Casamino acids-yeast extract medium and (4) McCarty's medium.

in the optical density of the reaction mixtures is plotted against the incubation period in hours. It will be seen that the modified McCarty's medium gives the best result, being the best medium for the production of the lytic enyme. It is of interest that while there is no significant difference in the growth in these media, the lytic activity of the culture filtrates varies greatly with the medium used. Fig. 2 shows the relationship between the lytic activity of culture filtrate of L_3 bacterium grown in modified McCarty's medium and the period of cultivation. The lytic activity against *C. diphtheriae* cell walls appears in the culture filtrate on the 4th day of incubation and reaches a maximum after 6 days incubation. No increase or decrease in the lytic activity of the filtrates occurred during the following 8 days cultivation.

2. *Concentration of the lytic factor from culture filtrates*

Seven day old culture fluid from 10 Roux's bottles (250 ml per bottle) was filtered through coarse filter paper. To the filtrate solid $(NH_4)_2SO_4$ was added to 0.6 saturation. The precipitate was removed by filtration through Celite (No. 503, John-Manvill Co., 1 g per liter). Solid $(NH_4)_2SO_4$ was added to the filtrate to saturation. The precipitate was collected by filtration on Celite as be before. Both precipitates were extracted in 50 ml of 0.1 M phosphate buffer, pH 8.0 and the extracts were separated from the Celite by filtration. The Celite was washed

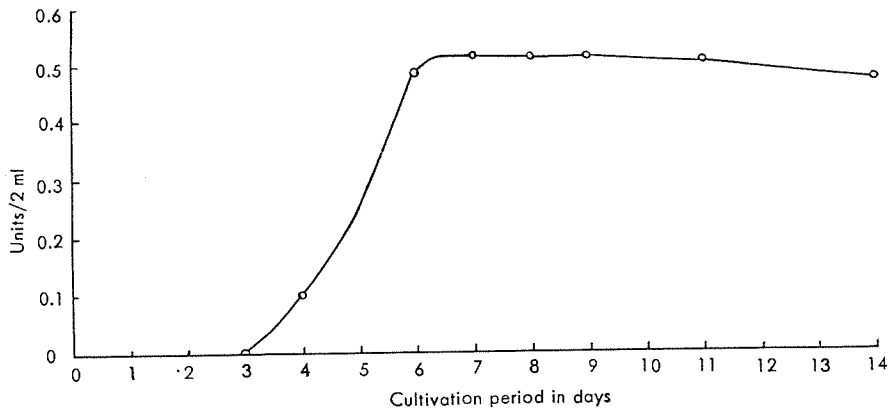

Fig. 2. Relationship between period of cultivation and lytic activity of culture filtrates against *C.diphtheriae* cell walls

three times with buffer and the combined filtrates were dialyzed against a large quantity of distilled water for 2 days at 4°C.

Table 1. Concentration of the lytic enzyme from culture filtrates by $(NH_4)_2SO_4$ fractionation

Fraction	Total units	Units/mg protein N
Original culture filtrate	500	6.4
Fraction precipitated at 0.6 saturation with $(NH_4)_2SO_4$	375	15.8
Fraction precipitated between 0.6 and 1.0 saturation with $(NH_4)_2SO_4$	30	2.9

The yields and specific activities per mg protein N in one preparation of this type are given in Table 1. Most of the lytic activity of the original culture filtrate (about 75 per cent) was recovered in the fraction precipitated at 0.6 saturation of $(NH_4)_2SO_4$. About 2.5-fold purification of the lytic factor has been effected by this process as indicated by the increase in the lytic activity per mg protein N.

Further purification of the lytic enzyme has been attempted using various absorbants, but satisfactory results have not yet been obtained. Further studies on this point are in progress.

3. *Heat stability and optimum pH*

Heat stability of the lytic factor was tested by heating samples of a 25-fold (by volume) concentrated culture filtrate, obtained as described above, at various temperatures for 30 minutes. As shown in Fig. 3, the lytic factor was almost completely inactivated by exposure to temperatures of 60°C or more. The lytic activity was considerably weakened by heating the preparation at 50°C for 30 minutes.

The lytic enzyme is highly heat-labile in this way, but it is surprisingly stable in a refrigerator and can be thus preserved for at least one month without any appreciable decrease in activity.

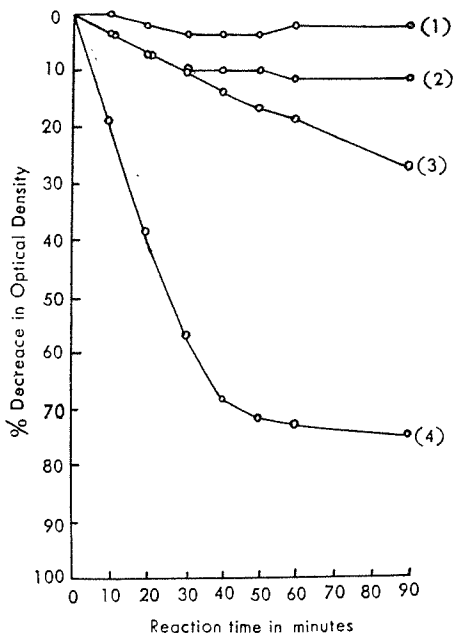

Fig. 3. Heat stability of the lytic enzyme of L_3 bacterium

The concentrated lytic enzyme solution was heated at pH 7.0 for 30 minutes at (1) 70°C, (2) 60°C, and (3) 50°C. Curve (4) indicate the non-heated control.

Fig. 4 presents the results of an experiment on the pH optimum for lysis of *C. diphtheriae* cell walls by the lytic enzyme. Tris- and phosphate-buffer (0.02 and 0.04 M final concentration, respectively) were used for the pH ranges 7.0–9.0 and 5.0–8.0. Although there is no sharp pH optimum, the lytic enzyme is much more active at an alkaline than at an acid pH and its activity is greatest between pH 7.5 and 8.5. It will also be seen from this figure that there is no appreciable difference of the lytic activity in different kind of buffer. It may be added in this connection that pH 7.0 buffer was used in other experiments, not because this pH is optimal for the lytic activity, but because it is a physiological pH.

4. *The activity range of the lytic enzyme*

The lytic activity of a 25-fold concentrete of the culture filtrate obtained as described above has been examined against isolated cell walls and intact cells of *C. diphtheriae*, BCG and *Staph. aureus* and intact cells of *M. lysodeikticus*, *S. lutea*, *S. valiabilis*, *Strep. pyogenes* and *B. megaterium*. As shown in Figs. 5 and 6, while a marked lysis of the cell walls and intact cells of *C. diphtheriae* (70 and 90 per cent reduction in optical density after 60 minutes incubation) was induced by the con-

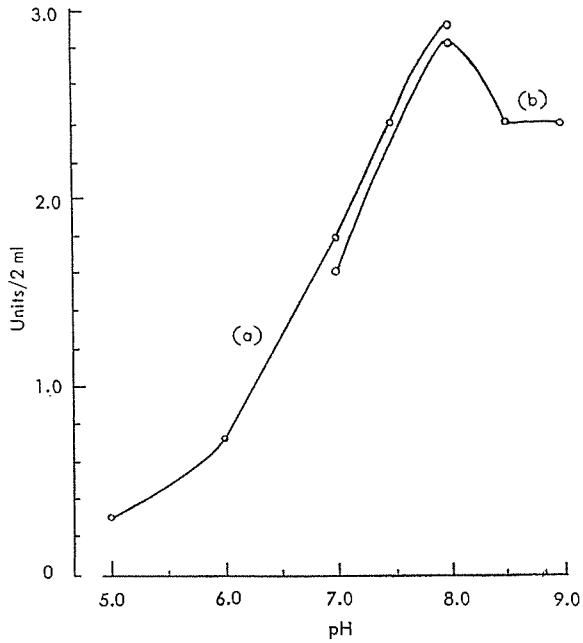

Fig. 4. The effect of pH of reaction mixtures on the activity of lytic enzyme

The lytic activity of a concentrated lytic enzyme solution was examined at various pH against C. *diphtheriae* cell walls, in the presence of (a) 0.04 M phosphate-buffer and (b) 0.02 M Tris-buffer.

centrated lytic filtrate used, none of the suspensions of other bacteria except *B. megaterium* showed any significant decrease in optical density under these experimental conditions. The curves given by control reaction mixtures containing intact cells but no lytic filtrate were ommitted from Fig. 6 to avoid complexity, but these controls did not show any appreciable reduction in optical density.

Figs. 7–10 illustrate the results of electron microscopic observation on the morphological changes induced by the lytic enzyme. It will be seen that the reduction in optical density of suspensions of *C. diphtheriae* cell walls or intact cells is really due to a dissolution of their structure.

5. *The chemical properties and the sensitivity to lysozyme and bacteral protease of C. diphtheriae cell walls*

Table 2 summarizes the results of a chemical analysis made on cell wall preparations isolated from sonically disrupted *C. diphtheriae*. It is shown that the chemical composition of *C. diphtheriae* cell walls is very similar to that of BCG cell walls both qualitatively and quantitatively (Kotani *et al.*, 1959c).

It has been demonstrted that the isolated cell wall preparations of *C. diphtheriae* are completely resistant to a crystalline egg white lysozyme (10 to 1,000γ/ml, a final concentration) and a crystalline protease produced by *Bacillus subtilis* (Nagarse, Teikoku Chemical Industry Co., 20 to 2,000 P.U.N./ml).

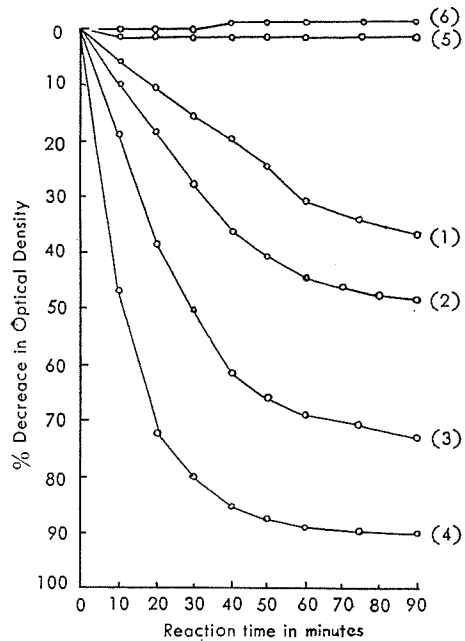

Fig. 5. The lysis of C. *diphtheriae* cell walls and intact cells by the lytic enzyme of L_3 bacterium

Curves (1) and (2) indicate the lysis of C. *diphtheriae* cell wall and intact cell suspensions by original culture filtrate of L_3 bacterium and (3) and (4) by the lytic factor concentrated by 0.6 saturation $(NH_4)_2SO_4$ (Concentrated 25-fold volume and 2.5-fold by the activity/mg protein by N). Curves (5) and (6) indicate the controls containing cell walls and intact cells, but no lytic enzyme.

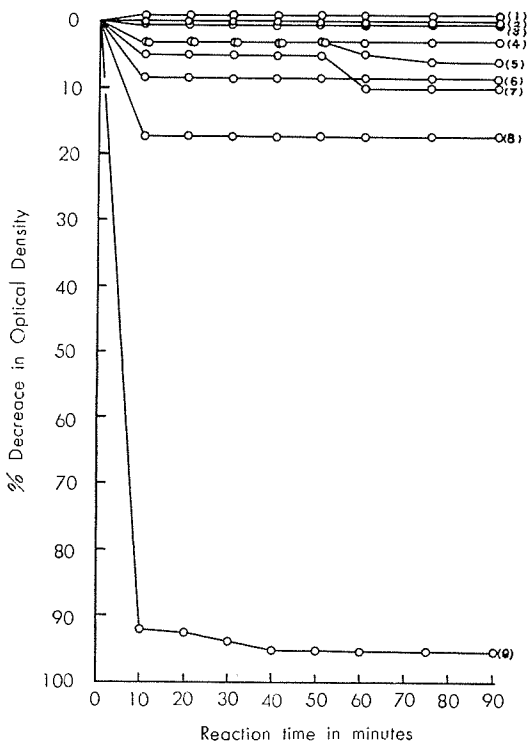

Fig. 6. The action of the lytic enzyme on intact cells and isolated cell walls of various bacteria

The concentrated lytic enzyme solution was incubated with the suspensions of (1) *Streptococcus pyogenes*, intact cells, (2) *Sarcina lutea*, intact cells, (3) *Staphylococcus aureus*, intact cells, (4) *Staphylococcus aureus*, cell walls, (5) *Micrococcus lysodeikticus*, intact cells, (6) *Sarcina valiabilis*, intact cells, (7) BCG, cell walls, (8) BCG, intact cells and (9) *Bacillus megaterium*, intact cells.

Table 2. Chemical composition of cell wall preparations of C. *diphtheriae*

Preparation examined	Total N %	Total P %	Sugars % *2,*3				
			Reducing substances	Hexose	Pentose	Methyl-pentose	Hexosamine
C. *diphtheriae* cell walls	4.8	0.19	35.1	9.8	23.8	1.0	7.1
BCG cell walls*1	3.0	0.36	21.2	10.2	11.7	1.2	3.7

Preparation examined	Lipids %				Principal sugars and amino acids detected in hydrolysates by paper chromatography
	Ethanol-ether soluble	Chloro-form soluble	Bound	Total	
C. *diphtheriae* cell walls	11.0	11.0	8.5	30.5	Arabinose, galactose, alanine, glutamic acid, meso-α,ε-diamino pimelic acid
BCG cell walls*1	17.2	21.4	22.5	61.1	〃 〃 〃

*1 Data from Kotani *et al.* (1959 c)

*2 Reducing substances, hexose, pentose, methylpentose and hexosamine are expressed as glucose, galactose, arabinose, rhamnose and glucosamine-HCl, respectively.

*3 Reducing substances, hexose, pentose and methylpentose, were determined after 2N H_2SO_4 hydrolysis for 2 hours at 100°C. Hexosamine was determined after 4N HCl hydrolysis for 10 hours at 100° C.

DISCUSSION

There is much literature on cell wall lytic principles produced by a variety of microorganisms (see the reviews of Salton, 1955b, 1960; Welsch, 1958). So far as the authors know, however, the work reported here is the first (with the possible exception of a study by Welsch, 1947) specifically designed to study the effect of the lytic enzyme active on the cell walls and intact cells of *C. diphtheriae*. Welsch reported in his short paper that the culture filtrate of *Streptomyces albus*, strain G, was capable of lysing intact cells of *C. diphtheriae*, but it cannot be decided from his descriptions whether the lysis observed was really due to lysis of the cell walls or to the acceleration of an autolytic process.

The lytic enzyme produced by L_3 bacterium studied in the present investigation is active against only *C. diphtheriae* and *B. megaterium* and does not exhibit any lytic activity against *M. lysodeikticus*, *S. lutea*, *S. valiabilis*, *Staph. aureus*, *Strep. pyogenes* and BCG. This specificity of the lytic activity indicates that the active factor concerned differs from the streptolytic enzyme (McCarty, 1952a, b). and actinolysopeptidase (Ghuysen, 1957) produced by *Streptomyces albus* and bacterial lysozymes produced by *B. subtilis* and other bacteria (Richmond, 1959a, b, c). Among the "autolytic" enzymes described in a variety of bacteria (Greenberg and Halvorson, 1955; Strange and Dark, 1957a, b; Nomura and Hosoda, 1956), there is no report on whether any of these enzymes exhibits a cell wall lytic activity against *C. diphtheriae*.

The study reported in this paper has not yet completed and further investigations are being carried out upon problems such as the purification of the lytic factor, analysis of the lytic process, protoplast formation by use of a purified lytic enzyme and so on. Further results will appear in this Journal.

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Fig. 7. Intact cells (control)

Fig. 8. Intact cells (lysed)

Fig. 9. Cell walls (control)

Fig. 10. Cell walls (lysed)

Figs. 7–10. Electron microscopic observations on the lysis of intact cells and cell walls of C. *diphtheriae*

Samples were taken from a 40-fold dilution of the reaction mixtures which were incubated at 37°C for 90 minutes. The samples were mounted on collodion films and shadowed with chromium. The shadowed preparations were examined with the Japan Electron Optics Laboratory electron microscope, Model JEM-5G. Scale : 1μ.