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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*

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SUMMARY

Call wall lysis of *Corynebacterium diphtheriae* by treatment with an enzyme produced by *Streptomyces sp.* was investigated in a reaction mixture containing various amounts of sodium succinate, sodium chloride and sucrose as stabilizers. Sodium succinate of 0.5 to 0.75 M final concentration was found to be the most satisfactory stabilizer for producing osmotically sensitive spherical bodies. The presence or absence of cell wall components on these bodies was examined by testing the agglutination and absorbing capacity of the ghosts derived from them and by measuring the rate and extent of release of serologically reactive cell wall components into the medium, using rabbit anti-cell wall serum. Evidence was obtained that only a trace, if any, of serologically reactive wall components remained on these bodies. It was thus demonstrated that "protoplasts" of *C. diphtheriae* were obtained by treatment of this organism with a purified cell wall lytic enzyme under suitable experimental conditions.

INTRODUCTION

Since the first description by Tomcsik and Guex-Holzer (1952) and by Weibull (1953) of the protoplasts of *Bacillus megatherium*, a considerable number of reports have been published on bacterial protoplasts. However, most of them concern the protoplasts released from non-pathogenic bacteria and only a few investigations have so far been made on the pathogens (Weibull, 1958; McQuillen, 1960).

In a previous paper (Mori *et al.*, 1960), the authors reported that the culture of a *Streptomyces sp.* isolated from soil according to the method of Salton (1955) exhibited a marked cell wall lytic activity against *C. diphtheriae* and that the lytic factor could be concentrated by fractionation of the culture filtrate with $(NH_4)_2SO_4$.

The present paper reports studies on the production of "protoplasts" of *C. diphtheriae* by treatment of the organisms with a partially purified preparation of this lytic enzyme. The results obtained clearly demonstrated that "proto-

* A preliminary report of this work was presented at the 8th Symposium on Bacterial Toxins, held at Kōyasan, Wakayama (July 21 and 22, 1961).

plasts" of *C. diphtheriae* could be produced by using the lytic enzyme under appropriate experimental conditions.

MATERIALS AND METHODS

1. *Cultivation of Streptomyces sp. producing lytic enzyme and preparation of a lytic culture filtrate*

Details of the methods used were given in the previous paper (Mori *et al.*, 1960).

2. *Perparation of cell walls of C. diphtheriae*

The methods employed for obtaining the cell wall preparations were essentially the same as those described by Kotani *et al.* (1959).

3. *Measurement of lysis of intact cells and isolated cell walls under the action of the lytic enzyme*

The rate and extent of lysis of intact cells and isolated cell walls of *C. diphtheriae* were determined by measuring the changes in optical density of the suspensions with a Hitachi photoelectric colorimeter, Type EPO-B. A No. 55 filter and test tubes of 18 ± 0.5 mm external diameter were employed in the determinations.

4. *Protein nitrogen determination*

The protein nitrogen content was estimated with the colorimetric method of Lowry *et al.* (1951) on the 10 per cent trichloroacetic acid insoluble fractions of the samples.

5. *Chromatographic resins*

Duolite A-2 and Duolite C-10 (Chemical Process Co., U.S.A.) were conditioned according to the descriptions of Okunuki *et al.* (1960) and of Hagihara *et al.* (1958), respectively. The acid form of A-2 resin and the sodium form of C-10 which were equilibrated with 0.2 M Na-phosphate buffer at pH 7.0 and 6.5 respectively were used in the experiments.

6. *Rabbit anti-cell wall serum*

Antiserum was produced by injection into rabbits of cell wall preparations suspended in an adjuvant consisting of liquid paraffin, Arlacel A (Atlas Powder Co., U.S.A.) and water in the ratio of 2:1:1 contaning streptomycin (500 μg/ml) and penicillin (1,000 units/ml). Healthy adult rabbits of mixed breed received two 0.8 ml injections of the antigen suspension (20 mg of cell wall preparations/ml) with a one week interval between injections: the antigen was divided into 4 portions of 0.2 ml each and each of them was subcutaneously injected into 4 sites on the back of the neck. The animals were bled 3 to 4 weeks after the second injection. The anti-cell wall serum thus obtained was preserved with NaN_3 (1:1,000) and stored in the refrigerator until needed.

7. *Serological techniques*

The precipitin reaction between the antiserum and lysed components of cell walls was performed by the ring test using narrow test tubes of 80 mm length and 4 mm internal diameter. The highest dilution of test antigens showing a positive reaction was determined to semiquantitate the content of serologically reactive substances. The reaction was carried out at room temperature for 5 hours. The precipitation was read at appropriate intervals and arbitrarily graded from − to 3+.

The agglutination reaction was carried out by mixing 0.5ml of cell wall suspension (optical density, about 0.3) and 0.5 ml of 2-fold serial dilutions of the test antiserum. The mixture was incubated at 37°C for 2 hours and then kept overnight in the cold. The agglutination of cell walls was read with the naked eye and was arbitrarily graded from − to 3+.

8. *Electron microscopic observations*

The techniques employed were essentially the same as those described by Kotani *et al.* (1959).

RESULTS

1. *Purification of the cell wall lytic enzyme*

A culture of *Streptomyces sp.* which had been incubated in McCarty's medium (McCarty, 1952) at 27°C for 7 to 9 days was filtered through coarse filter paper. The dark brown filtrate was then decolorized by passing it through a Duolite A-2 column at a flow rate of 10 to 20 ml per minute. A 3×20 cm column of the resin was adequate for treatment of 10 l of filtrate. Seventy to 90 per cent of the colored materials were removed by this procedure with 15 to 40 per cent loss of enzyme activity.

A 10 l batch of decolorized culture filtrate was mixed with solid $(NH_4)_2SO_4$ until 0.3 saturation was attained. The mixture was stored overnight in the cold. The resulting precipitate was filtered off and to the filtrate was added solid $(NH_4)_2SO_4$ to 0.8 saturation. After standing the mixture overnight in the cold, the precipitate was collected by filtration and dissolved into about 100 ml of water. The combined concentrate from several batches was dialysed against water for 24 hours in the cold and then was mixed with an equal volume of 0.2 M Na-phos-

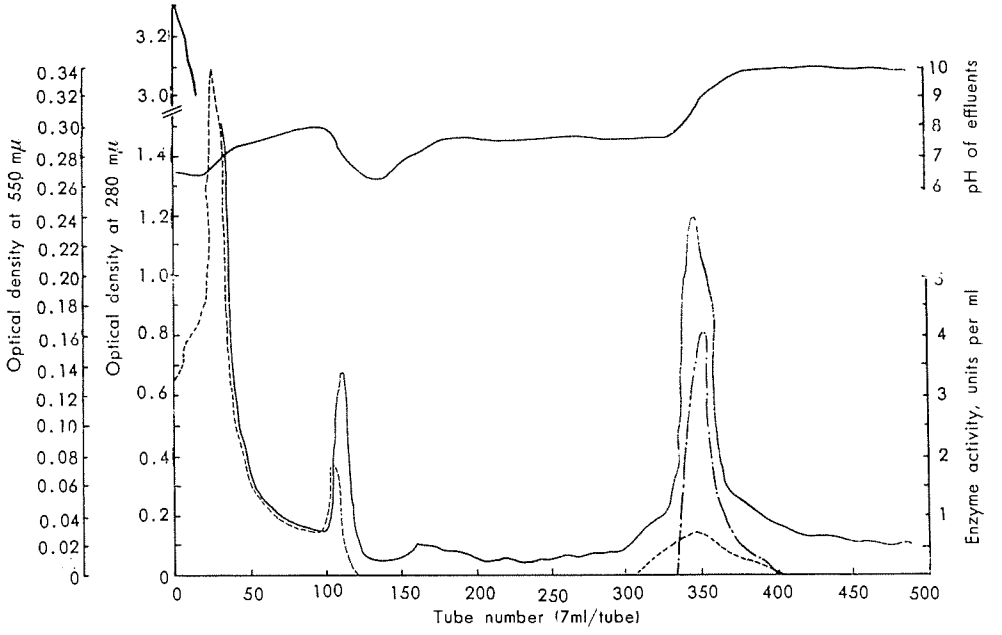

Fig. 1. Chromatographic Purification of a Lytic Culture Filtrate Concentrated by Precipitation with $(NH_4)_2SO_4$ on a 3×10 Column of Duolite C-10 (Sodium Form)

Elution was performed with a mixture of 0.05 M Na-phosphate buffer (pH 8.0) and 0.5 M NaCl and then with a mixture of 0.05 M Na_2HPO_4, 0.05 M NH_4OH and 0.5 M NaCl.

— - — ; cell wall lytic activity (units/ml)
..... ; optical density at 280 mμ (a lower solid line)
- - - - ; optical density at 550 mμ

phate buffer (pH 6.5). The mixture was filtered to remove insoluble materials. All filtration procedures were carried out with the aid of Celite No. 503 (Johns-Manville Products, U.S.A.). The filtered concentrate (about 800 ml in the experiment presented in Table 1) was then passed through a Duolite C-10 column (3×40 cm) at a flow rate of about 5 ml per minute and the column was rinsed with 1 l of water. While the active principle was almost completely adsorbed onto the column, the bulk of inactive proteins and colored materials was not. Elution was performed with about 1 l of a mixture of 0.05 M Na-phosphate buffer (pH 8.0) and 0.5 M NaCl followed by about 2 l of a mixture of 0.05 M Na_2HPO_4, 0.05 M NH_4OH and 0.5 M NaCl, at a flow rate of about 5 ml per minute. The effluent was collected in 7 ml fractions. Each fraction was surveyed by determination of its cell wall lytic activity and of its absorption at 280 mμ for protein and at about 550 mμ for colored materials (using a Shimazu spectrophotometer, Model QB-50, and a Hitachi photoelectric colorimeter, Type EPO-B, with a No. 55 filter respectively). As shown in the elution curve of the typical experiment presented in Fig. 1, the cell wall lytic enzyme appeared in the effluent of the second alkaline mixture, and its appearence was accompanied by a sharp rise in pH. All procedures with Duolite C-10 were carried out in the cold and as rapidly as possible.

Table 1 summarizes the results of a representative concentration and purification experiment in terms of the percentage recovery and purification at each individual step. An overall purification of about 25-fold was achieved with about 10 per cent recovery.

Table 1. Summary of Concentration and Purification Procedures

Fraction	Total volume (ml)	Total units	Total protein (mg N)	Specific activity (units/mg N)	Colored materials (O.D. at 550 mμ)	Recovery (per cent)
Crude culture filtrate	10,550	4,457	160.4	27.9	0.4	100
Filtrate passed through Duolite A-2	10,550	2,775	76.0	36.5	0.05	62
$(NH_4)_2SO_4$ fraction (0.3-0.8 saturation)	114	1,220	17.6	69.3	0.55	27
$(NH_4)_2SO_4$ fraction before chromatography	770	3,985	55.5	71.8	0.18	100
Eluate from Duolite C-10 (tubes No. 340-364)	195	1,521	2.2	691.4	0.015	38

In some of the experiments, the combined active fractions released from the Duolite C-10 column were saturated with solid $(NH_4)_2SO_4$ to concentrate the enzyme. During dialysis against water, a dark brown precipitate settled out. After separation of this precipitate, 85 per cent of the lytic activity remained in the slightly yellowish supernatant fluid. An enzyme preparation with a specific activity of 1,560 units per mg protein nitrogen was thus obtained in one of the experiments.

The enzyme preparations purified as described above (with or without the final purification step) were used in the following study.

2. *Production of "protoplasts" of C. diphtheriae by treatment with the purified enzyme*

A substrain of Park Williams No. 8 Toronto Harvard strain of *C. diphtheriae* was used throughout the present experiments. A 24 hour old culture on a Loeffler's slant was first inoculated into 150 ml of modified Pope and Smith medium (Yoneda and Matsuda, 1961) containing 0.2 μg iron per ml and incubated at 35°C for 24 hours with shaking at 120 strokes per minute at an amplitude of 7 cm. One ml of the growing culture was reinoculated into another 150 ml of the same medium and incubated with shaking as described above. A 20 hour old culture (optical density, about 6.0), the filtrate of which contained 50-100 Lf per ml, was harvested by centrifugation. The sedimented cells were resuspended into the original amount of water. The cell suspension thus obtained was used in the following experiments.

1) *Selection of a stabilizing medium suitable for experiments on protoplast formation*

The relative merits of Na-succinate, NaCl and sucrose as stabilizers were

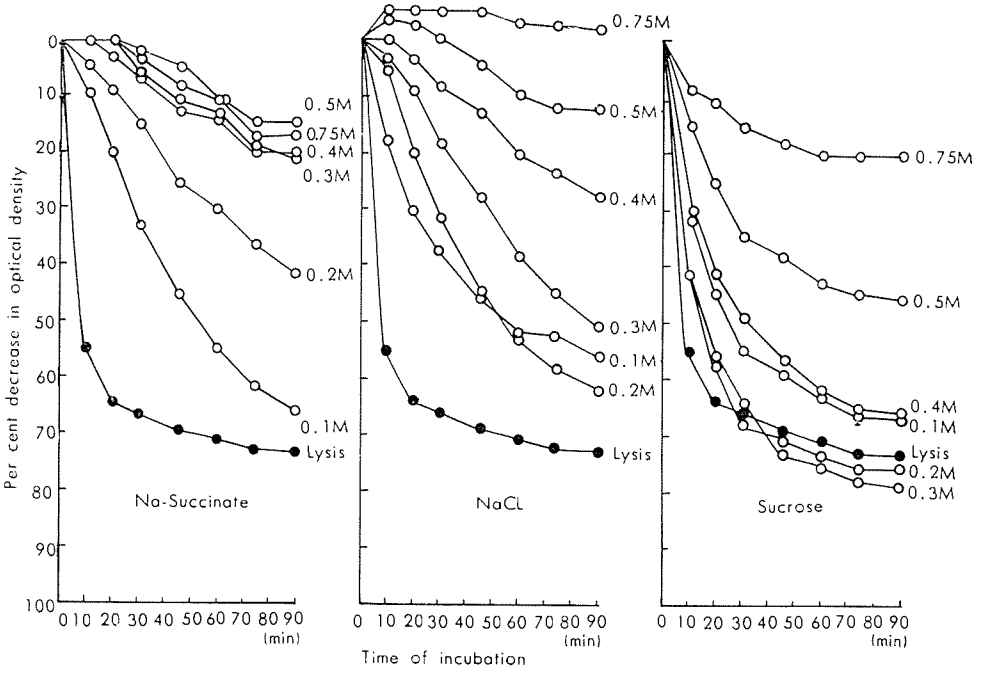

Fig. 2. Influence of the Supporting Medium on Lysis of Cells of C. *diphtheriae* on Incubation with the Lytic Enzyme

Reaction mixtures consisted of cell suspension (optical density, about 0.6), lytic enzyme (12.5 units/ml), stabilizers of various concentrations, 0.002 M $MgSO_4$ and 0.02 M tris-buffer (pH 8.0). Optical density was read after various periods of incubation at 37° C.

compared in the following way. The reaction mixtures consisted of 0.4 ml of a cell suspension of *C. diphtheriae* (optical density, about 6.0), 2.0 ml of lytic enzyme solution (25 units/ml) and 1.6 ml of solutions of the test stabilizers at various concentrations in 0.05 M tris-buffer (pH 8.0) containing 0.005 M $MgSO_4$. The mixtures were incubated at 37°C in a water bath for 90 minutes and the changes in optical density were followed at intervals.

The results are summarized in Fig. 2. It may be seen that a marked reduction in optical density, due to lysis of the cells by enzyme action, was seen in the control reaction mixture containing no stabilizers and that the optical density reduction was markedly inhibited by the presence of sufficiently high concentrations of stabilizers. As regards the relative merits of the stabilizing capacities of the three solutes examined, it should be pointed out that there were some differences in their stabilizing effects at different concentrations: while the stabilizing effect of Na-succinate was fairly constant over a wide range of concentration, 0.3 to 0.75 M, in the case of both NaCl and sucrose a marked decrease in stabilizing effect was found as the concentration was reduced from 0.75 to 0.3 M. Thus 0.75 M NaCl had a stronger stabilizing effect than 0.75 M Na-succinate, but the latter was more effective than the former at a concentration of 0.3 M.

The next step in the investigation was to obtain evidence that the observed effect of the stabilizers was not attributable to an inhibitory effect on the cell wall lytic enzyme. For this purpose, lysis of isolated cell wall preparations by the lytic enzyme (12.5 units/ml) was investigated in the presence and absence of the

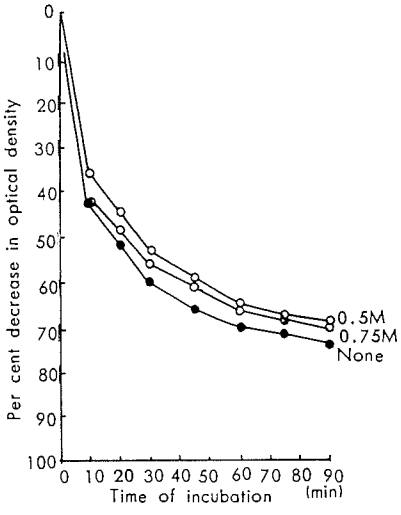

Fig. 3. Influence of a High Concentration of Na-Succinate on Lysis of the Cell Walls on Incubation with the Lytic Enzyme

A cell wall suspension was incubated at 37° C with the lytic enzyme (12.5 untis/ml) in the presence or absence of Na-succinate.

stabilizers. Fig. 3 presents the results of an experiment in which the reduction in optical density of a cell wall suspension in the presence of 0.5 and 0.75 M Na-succinate was compared with that in the absence of the stabilizer. The rate and extent of release of cell wall components which reacted with anti-cell wall serum were also investigated, as shown in Table 2. It may be concluded that Na-succinate, at least at 0.5 M final concentration, exhibited only a slight inhibitory influence on lysis of cell walls by the lytic enzyme. Silmiar results were obtained with NaCl.

Table 2. Influence of a High Concentration of Na-Succinate on Release of Serologically Reactive Components from the Cell Walls on Incubation with the Lytic Enzyme (12.5 Units/ml)

Period of incubation with the lytic enzyme (minutes)	Presence or absence of Na-succinate (0.5 M)	Dilution of the supernatants of reaction mixtures						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
5	+	+*	+	±	−	−	−	−
	−	2+	2+	2+	+	−	−	−
30	+	2+	2+	2+	2+	−	−	−
	−	2+	2+	2+	2+	−	−	−
90	+	3+	3+	2+	2+	2+	+	−
	−	3+	3+	3+	2+	2+	+	−

* Readings of the precipitin reaction with an undiluted anti-cell wall serum (ring test).

The obaservations described in the foregoing paragraphs indicate that both Na-succinate and NaCl (and presumably sucrose, too) can be used as stabilizers in experiments on the production of protoplasts of *C. diphtheriae* with the lytic enzyme. Since the effective, stabilizing concentration of Na-succinate was lower than that of NaCl, Na-succinate was chosen as stabilizer in the following experiments.* It may be said in this connection that use of a stabilizer which is effective at a low concentration may be more convenient in future investigations on protoplasts.

2) *Observations with a phase contrast microscope*

Drops of the reaction mixture containing 6 units per ml of the lytic enzyme and 0.75 M Na-succinate were put onto a mcroscopic slide and covered with a cover glass. The morphological changes of the cells were followed at 25°C for 210 minutes under a phase contrast microscope. Fig. 4 illustrates the results of this study. It can be seen from this figure that the bacillary form of *C. diphtheriae* changed to a spherical form under the action of the lytic enzyme in the presence

* It is worth mentioning in this connection that a definite, spontaneous agglutination of bacterial bodies was observed during incubation of the cells with the lytic enzyme in the presence of Na-succinate of higher concentrations. The protein content of the supernatant from a reaction mixture containing 0.75 M Na-succinate indicated that no signficant amount of cytoplasmic protein was released from the cells by enzyme action. This finding seems to justify the assumption that about 20 per cent optical density reduction observed (cf. Figs. 2 and 5) is not due to real lysis of the cells, but mainly due to their agglutination by Na-succinate. No similar agglutination of the bacterial bodies was seen in reaction mixtures containing NaCl or sucrose as stabilizers.

of stabilizer. Photograph B in Fig. 4 shows chains consisting of a few spherical forms seen during incubation. This is of some interest in connection with the report of Davis and Mudd (1955) that there are septa in some *C. diphtheriae* cells.

3) *Osmotic sensitivity of the spherical bodies and release of serologically reactive cell wall components into the medium*

A reaction mixture containing 12.5 units per ml of lytic enzyme and 0.5 M Na-succinate was incubated at 37°C and aliquots of the mixture were withdrawn and centrifuged at intervals. The pellet obtained was resuspended in the original volume of water to examine the osmotic sensitivity of the sedimented cells which had been incubated with the lytic enzyme for various periods. The extent of decrease in optical density resulting from this treatment is shown in Fig. 5, together with curves illustrating the changes in optical density of the reaction mixtures in the presence and absence of the stabilizer. It is readily seen that cells incubated with the enzyme for only 5 minutes showed a marked osmotic sensitivity and burst on resuspension in water.

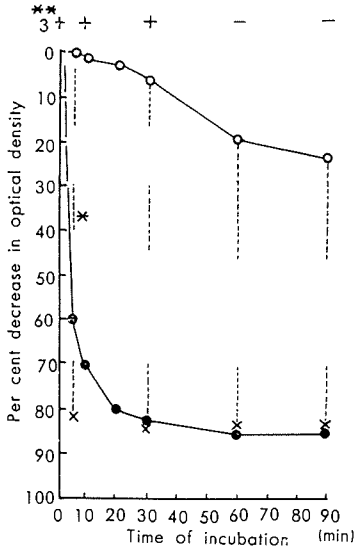

Fig. 5. Osmotic Sensitivity of the Cells of C. *diphtheriae* Incubated with the Lytic Enzyme (12.5 Units/ml) in the Presence of 0.5 M Na-Succinate and Agglutination of the Ghosts by Anti-Cell Wall Serum

Curves indicate the changes in optical density of the reaction mixtures during incubation with the lytyic enzyme in the presence (○) and absence (●) of Na-succinate.

* Optical density reduction produced by resuspending the sedimented cells in the original volume of water.

** Agglutination of the ghosts on incubation with anti-cell wall antiserum. Control intact cells show 3+agglutination. Readings were made after 2 hours incubation at 37° C.

In parallel with the above analysis, the release of serologically reactive cell

Table 3. Release of Serologically Reactive Cell Wall Components from Cells during Incubation with the Lytic Enzyme (12.5 Units/ml) in the Presence or Absence of 0.5 M Na-Succinate

Period of incubation with the lytic enzyme (minutes)	Presence or absence of Na-succinate (0.5 M)	Dilution of the supernatants of reaction mixtures						
		1:1	1:2	1:4	1:8	1:16	1:32	1:64
5	+**	2+*	±	—	—	—	—	—
	—***		3+	2+	2+	±	—	—
30	+	3+	2+	2+	+	—	—	—
	—		3+	2+	2+	+	—	—
90	+	3+	2+	3+	3+	2+	±	—
	—		3+	3+	2+	2+	—	—

* Readings of the precipitin reaction with an undiluted anti-cell wall serum (ring test).

** For "protoplasts" formation.

*** For complete lysis.

wall components into the medium was also studied by the precipitin test with rabbit anti-cell wall serum, in the superpernatant fluids from the aliquots withdrawn at intervals, as shown in Table 3. The rate of release of cell wall components which reacted with the antiserum was somewhat retarded in the presence of the stabilizer, but the content of the reactive components in the supernatants of the mixtures incubated for 90 minutes was almost the same irrespective of the presence or absence of stabilizer.

4) *Electron microscopic observation of the pellet fraction*

A reaction mixture which contained 8 units per ml of enzyme and 0.75 M of Na-succinate was incubated for 90 minutes. After centrifugation, the pellet fraction was suspended in 1 per cent OsO_4 solution in 0.025 M tris-buffer (pH 7.0) and kept overnight in the cold. The pellet fraction which was burst and fixed simultaneously in this way was washed with water and mounted on collodion films for electron microscopy. Fig. 6 shows that the pellet fraction treated as described above consists of thin membraneous structures with some internal contents, namely of ghosts.

5) *Some serological properties of the ghosts in relation to anti-cell wall serum*

The ghost suspension obtained in the experiments described under section 3) was centrifuged at 10,000 *g* for 20 minutes. The ghosts fraction which sedimented was washed once with 0.85 per cent saline and resuspended in the original volume of saline. The agglutination of the ghosts thus obtained was examined by mixing 0.2 ml of undiluted anti-cell wall serum and 0.8 ml of the suspension and then incubating the mixture at 37°C for 2 hours. The ghosts from reaction mixtures incubated for 5 and 30 minutes were definitely agglutinated by the antiserum although to a far less extent than intact cells. On the other hand, ghosts obtained after 60 and 90 minute incubation did not show any appreciable agglutination (Fig. 5). These findings indicate that osmotic sensitivity of treated

cells did not always mean a complete absence of serologically reactive cell wall components on them and that in order to obtain bacterial bodies deprived of all serologically reactive cell wall components, rather prolonged incubation with the lytic enzyme was required.

The absence of serologically reactive cell wall components on ghosts obtained after 90 minute incubation was further examined by testing their ability to absorb anti-cell wall antibody. Absorption was made in the following manner. Two ml of the ghost suspension, derived from 4 ml of the reaction mixture, was added to 1 ml of anti-cell wall serum. After incubation at 37°C for 2 hours and overnight in the cold, the mixture was centrifuged and the supernatant fluid was separated (once absorbed antiserum). One and half ml of this abosorbed antiserum was reabsorbed with another 1 ml of the ghost suspension from 4 ml of reaction mixture (twice absorbed antiserum). Absorption of the antiserum was also performed with supernatant fluid separated from the pellet fraction (cell wall lysates). Half ml of the antiserum was mixed with a 2 ml of the superntant fluid and the mixture was treated in the same manner as described above. The antiserum absorbed in this way was heated at 60°C for 1 hour to inactivate cell wall lytic enzyme derived from the supernatant fluid. Two controls were set up: control 1 was a 1:5 saline dilution of antiserum and control 2 was antiserum which was mixed with lytic enzyme and stabilizer, and which received the same treatment as in the antiserum absorbed with the supernatant fraction. Volumes of 0.5 ml of serial 2-fold dilutions from 1:5 to 1:80 were made of each of the absorbed and control antisera and they were mixed with an equal volume of cell wall suspension. Agglutination was read after incubation for 2 hours at 37°C and overnight storage in the cold room. The results are summarized in Table 4. It can be seen from this table that the ghosts from a reaction mixture incubat-

Table 4. Absorbing Capacity for Anti-Cell Wall Antibody of the Ghosts and Supernatants Derived from a Reaction Mixture Incubated with the Lytic Enzyme (12.5 Units/ml) in the presence of 0.5 M Na-Succinate

Anti-cell wall serum absorbed with	Dilution of absorbed and control antiserum				
	1:10	1:20	1:40	1:80	1:160
Ghosts (once)	3+	3+	2+	±	—
Ghosts (twice)	3+	2+	+	±	—
Supernatants**	—	—	—	—	—
None: Control 1	3+	3+	2+	±	—
Control 2**	3+	3+	+	±	—

* Readings of the agglutination with a cell wall suspension. The reactions were read after incubation at 37°C for 2 hours and overnight storage in the cold.

** To inactivate the remaining cell wall lytic enzyme, these antisera were heated at 60°C for 1 hour.

ed for 90 minutes did not absorb significant amounts of anti-cell wall antibody

even after repeating absorption treatments, in sharp contrast to the quite strong absorbing activity of the supernatant fraction derived from the same reaction mixture. It should be added in this connection that there was no significant difference in the agglutinin titer of controls 1 and 2, indicating that treatment of the antiserum with the cell wall lytic enzyme and heat did not affect the antibody titer appreciably.

DISCUSSION

Many studies have been made on the protoplasts of non-pathogenic bacteria from various points of view and a great deal of valuable information has been accumulated. However, there have been few studies on pathogens and only those of Freimer, Krause and McCarty (1959) on L forms and protoplasts of group A *streptococci* produced by use of *Streptomyces albus* enzyme and phage-associated lysin and those of Mitchell and Moyle (1957) and of Kato *et al.* (1960) on *Staphylococcus aureus* are available in the literature. Mitchell and Moyle reported an autolytic release of "protoplasts" and the latter investigators studied "protoplasts" formation with a cell wall lytic enzyme produced by a *Flavobacterium sp.* isolated in our laboratory. There have apparently been no reports on the protoplasts of *C. diphtheriae.*

The results obtained in this work clearly indicate that osmotically sensitive spherical bodies, which are almost completely devoid of serologically reactive cell wall components, can be produced from *C. diphtheriae* cells by treatment of the organism with particular cell wall lytic enzyme in the presence of an appropriate stabilizer. It is uncertain whether there are any cell wall components in the spherical bodies obtained, because serologically inactive cell wall components may remain on the surface of the bodies. Therefore, the use of the term <protoplasts without quotation marks> should be reserved for the time when more informations are available on the chemical properties of the ghosts derived from the "protoplasts", in view of the recommendation by Brenner *et al.* (1958). Futhermore, since some fraction of the cell population may escape the action of the lytic enzyme, the efficiency of "protoplasts" formation by the method reported here must await further investigations.

At any rate the establishment of a method for production of "protoplasts" of *C. diphtheriae* would provide a very useful tool for investigation of various problems on this organism, especially for elucidation of the mode of production of its exotoxin.

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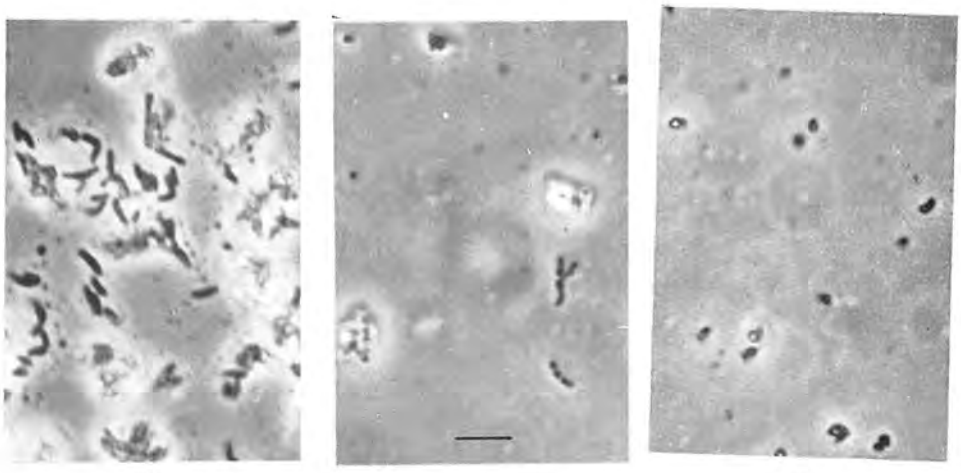
EXPLANATION OF PHOTOGRAPHS

Fig. 4. Changes of *C. diphtheriae* into Spherical Bodies on Incubation with the Lytic Enzyme in the Presence of Na-succinate

Cells of *C. diphtheriae* were incubated with the lytic enzyme (6 units/ml) in the presence of 0.75 M Na-succinate at 25° C. Morphological changes of the cells were followed under a phase contrast microscope. (A) Cells before incubation, (B) cells incubated for 55 minutes (chains consisting of a few spherical forms are shown) and (C) cells incubated for 210 minutes. Scale : 5 μ.

Fig. 6. Electron Microscopic Appearance of the Ghosts of *C. diphtheriae*

Intact cells of *C. diphtheriae* were incubated with the lytic enzyme (8 units/ml) in the presence of 0.75 M Na-succinate, at 37° C for 90 minutes. The sedimented cells were burst and fixed simultaneously by being resuspended in 1 per cent OsO_4 solution in 0.025 M tris-buffer (pH 7.0). The burst cells were mounted on collodion films and shadowed with chromium. The preparations were examined with a Japan Electron Optics Laboratory Electron Microscope, Model JEM-5G. Scale: 1 μ.



(A) (B) (C)

Fig. 4.

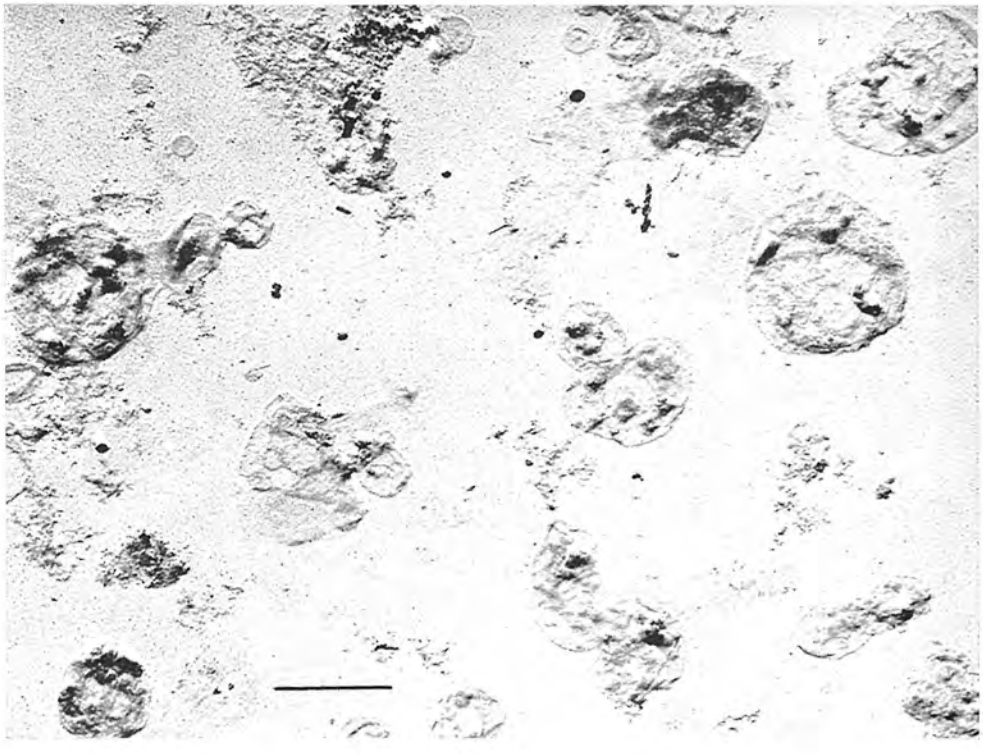


Fig. 6.