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PREPARATION OF CYTOPLASMIC MEMBRANES OF *STAPHYLOCOCCUS AUREUS* FDA 209P THROUGH PROTOPLASTS MADE WITH THE L-11 ENZYME AND A PRELIMINARY ANALYSIS OF MEMBRANE ANTIGENS

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SUMMARY Studies were made on the optimal experimental conditions for production of the protoplasts of *Staphylococcus aureus* FDA 209P using *Flavobacterium* L-11 enzyme. Treatment of cells in the early or mid stage of the stationary phase with enzyme in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.75 M sucrose at 37 C for two hr resulted in reproducible formation of stable protoplasts. A cytoplasmic membrane fraction virtually uncontaminated with cell wall peptidoglycan was isolated by rupture of the protoplasts and digestion of the resulting ghosts with nucleases. The membranes were " solubilized " by sonication in alkaline solution, and their antigens were analyzed and compared with those of dialyzed lyzates of the cell wall and the cytoplasmic fraction by the immunodiffusion (Ouchterlony) method. The " solubilized " membranes gave four or five precipitation lines on reaction with rabbit antiserum prepared against 209P whole cells. The line closest to the antigen well seemed to represent a nonimmunological reaction since lines fusing with this line were given by many preimmune rabbit sera as well as antisera against bacteria other than *S. aureus*. The second precipitation line from the antiserum well was found to be due to ribitol teichoic acid attached onto the membranes from the cell wall lyzates. The remaining two or three precipitation lines were tentatively assumed to be due to specific antigens of the cytoplasmic membranes, though the one nearest to the antiserum well fused with one of the precipitation lines produced by the cytoplasmic fraction. The possibility that membrane antigens with a protein nature were destroyed during formation of protoplasts by protease(s) present in the L-11 enzyme preparation and so were lost from the membrane preparation used in this study was discussed.

INTRODUCTION

Tomcsik and Guex-Holzer (1954) first presented evidence that the cytoplasmic membranes of bacterial cells contain antigens distinguishable from those in other parts of the cells. They demonstrated with *Bacillus megaterium*, strain M, that rabbit antiserum prepared against the free protoplasts agglutinated protoplasts in moderate dilutions, but did not agglutinate the cell wall suspension, while antiserum against the cell walls behaved in the reverse way. In a subsequent study (Tomcsik and Guex-Holzer—quoted by Tomcsik, 1956) using the agglutination and complement fixation tests they showed that isolated and washed cytoplasmic membranes behaved like whole protoplasts serologically. The findings of Tomcsik and Guex-Holzer were confirmed and extended by Vennes and Gerhardt (1956) who compared the cells, protoplasts, cell walls and cytoplasmic membranes of *B. megaterium*, strain KM, immunologically using the tube agglutination test, complement fixation test and cross-absorption method. The particle fraction originating from the cytoplasmic membranes was also found to contain a specific antigen(s) through studies on the immunological properties of the cell wall-, particle-, and soluble cytoplasmic-fractions isolated from BCG cells after sonic disruption (Kotani et al., 1960).

On the other hand, Zabriskie and Freimer (1966) described a serological cross reaction between group A streptococcal cytoplasmic membranes and human cardiac myofibers as well as smooth muscle of vessel walls, and suggested that this cross reaction was related to the pathogenesis of rheumatic fever. Further Rapaport, Chase and Solowey (1966) showed that the cytoplasmic membrane components of group A streptococci could induce skin allograft sensitivity in mice and other mammals, and that *Staphylococcus aureus* and *Staphylococcus epidermidis*, but not other gram-positive or gram-negative bacteria, also had this activity. On the other hand, Whiteside and Salton (1970) reported a detailed investigation on the antigenic nature of Ca^{2+}-activated adenosine triphosphatase isolated and purified from the membranes of *Micrococcus lysodeikticus*. Analysis of the antigens in bacterial cytoplasmic membranes seems to be an attractive subject for both medical and general microbiological studies.

Preparation of cytoplasmic membranes in a sufficiently pure and intact state is a prerequisite for successful analysis of their antigens. This is most satisfactorily achieved using appropriate lytic enzymes against the cell walls of the bacteria in question, though it is possible to isolate a fraction with the chemical and serological properties of cytoplasmic membranes from cells after mechanical disintegration. The present report is on the production of protoplasts of *S. aureus* FDA 209P using *Flavobacterium* L-11 enzyme, the isolation of cytoplasmic membranes and analysis of their antigens. A short communication on formation of "protoplasts" of *S. aureus* (strain Newman 1) with the L-11 enzyme has already been presented (Kato et al., 1960).

MATERIALS AND METHODS

1. *Organism and growth conditions*

Protoplasts were prepared from *S. aureus* FDA 209P. This organism was maintained on slants of nutrient phosphate broth (Nissui Seiyaku Co., Tokyo) solidified with 1.2% agar. An L-shaped test tube containing 5 ml of nutrient phosphate broth supplemented with 0.5% glucose was inoculated with one loopful of an 18 hr slant culture and incubated at 37 C on a reciprocal shaker oscillating at 100 strokes/min at an amplitude of 7 cm. Volumes of 1 ml of a 5 to 6 hr broth culture were inoculated into long-necked, flat-bottom, round flasks (650 ml volume) containing 100 ml of the above liquid medium. Growth of the test organisms was followed turbidmetrically at 550 mμ with a Shimadzu-Baush & Lomb Spectronic 20 colorimeter (Shimadzu Seisakusho Ltd., Kyoto). After appropriate periods of incubation, the organisms were harvested by centrifugation at $3,000 \times g$ for 10 min, and washed with deionized water. Harvesting and

washing were carried out in the cold to minimize possible autolysis of the cells. Protoplasts were prepared from washed cells within a few hours of harvesting.

2. *Staphylolytic enzyme preparation*

A crude enzyme preparation (lot 32) produced in the Fuji pilot plant (Mishima, Shizuoka) of the Kyowa Fermentation Industry (Tokyo) was partially purified as follows. Five g of the crude enzyme were dissolved in 200 ml of 0.01 M potassium phosphate buffer, pH 8.0, and the solution was filtered through sufficient CM-cellulose (SERVA Entiwicklungslabor, West Germany) equilibrated with the above buffer on a sintered glass filter. The cellulose was eluted with 0.2 M potassium phosphate buffer, pH 8.0, and fractions (50 ml each) of the almost colorless effluent were collected. Aliquots (10 μl) of the fractions were assayed for staphylolytic activity by incubating them with whole cells of *S. aureus*, strain Copenhagen, in 2.4 ml of 0.01 M potassium phosphate buffer, pH 8.0 at 37 C for one hr. The final concentration of cells was 0.25 mg dry weight/ml. Active fractions were pooled and thoroughly dialyzed against deionized water. The lytic activity of a partially purified enzyme solution (420 ml) thus obtained was assayed on cell walls and whole cells of *S. aureus* (strain Copenhagen) in 0.01 M potassium phosphate buffers, pH 7.2 or 8.0. One unit of lytic activity is defined as previously described (Suginaka et al., 1967). The partially purified enzyme preparation had activities of 100 and 1,200 units/ml with cell walls and whole cells respectively, at pH 8.0. At pH 7.2, the activity with cell walls was only 33 lytic units/ml.

3. *Specimens of teichoic acid and protein A*

A purified preparation of ribitol teichoic acid was isolated from the cell walls of *S. aureus* (strain Copenhagen) by extraction with cold 5% trichloroacetic acid, treatment of the crude material with the L-11 enzyme to remove the attached peptidoglycan, and fractionation of the enzymatic digests by gel filtration. Details of the procedure were described previously (Murayama, Kotani and Kato, 1968). Cell walls of strain Copenhagen were used as a source for isolation of the reference ribitol teichoic acid specimen, since Torii, Kabat and Bezer (1964) showed that the cell walls of this strain contained both an α-linked *N*-acetylglucosaminyl-ribitol polymer and a β-linked *N*-acetylglucosaminyl-ribitol polymer. An authentic specimen of protein A was generously given by Dr. A. Forsgren, the Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden.

4. *Chemical analyses*

Total hexosamines, total phosphorus and protein were determined by the methods of Ghuysen, Tipper and Strominger (1966), Lowry et al. (1954) and Lowry et al. (1951), respectively. D-amino acids were measured following the procedures described by Ghuysen, Tipper and Strominger (1966) using D-amino acid oxidase prepared by the method of Massey, Palmer and Bennet (1961). Amino acid and amino sugar analyses were performed on specimens hydrolyzed with 6 N HCl at 100 C for 14 hr, using a Hitachi KLA-3B Amino Acid Analyzer (Hitachi Ltd., Tokyo). Ultraviolet absorption measurements were made in a Hitachi Recording, Model EPS-3T, Spectrophotometer with a 10 mm light path.

5. *Preparation of rabbit antisera*

Cells of *S. aureus* FDA 209P grown overnight as already described were washed thoroughly with deionized water and lyophilized. Five mg of lyophilized cells were suspended in 1.2 ml of deionized water, and heated at 60 C for one hr. The suspension was mixed with 0.4 ml each of solutions of penicillin G (20,000 units/ml) and streptomycin hydrochloride (250 mg/ml), and vigorously homogenized with an incomplete Freund's adjuvant consisting of 4 ml of Drakeol (Pennsylvania Oil Co., Penn., USA) and 2 ml of Arlacel A (Atlas Powder Co., Ind., USA). One ml portions of the resulting immunizing antigen were injected into the footpads (0.25 ml/pad) of adult rabbits weighing about 3 kg, 3- or 4-times at weekly intervals. The rabbits received one intravenous booster injection of 2 ml of a heat-killed cell suspension (2 mg dry weight/ml) on day 21 or 28. One week after the last injection, the animals were exsanguinated. The immune serum specimens obtained were mixed with sodium azide at a final concentration of 0.1% and stored at 4 C.

A similar immunization schedule was adopted to obtain rabbit antisera against *S. epidermidis* (ATCC 155), *Micrococcus lysodeikticus* (NCTC 2665), *Gaffkya tetragena* (ATCC 15292) and *Sarcina lutea* (IFO 3232). Rabbit antiserum specimens against Group A *Streptococcus pyogenes* (type 12, strain S. F. 42) and Group A-variant *S. pyogenes*

(type 1, K43 var.) were supplied by Drs. S. Hamada and T. Narita of this laboratory and prepared by repeated intravenous injections of heat-killed cells according to the schedule for anti-C carbohydrate antibody production.

6. *Immunodiffusion*

A micromodification of the two dimensional, double diffusion technique of Kabat and Mayer (1961) was performed in agar gel. About 7 ml portions of 1.0–1.2% agar (Difco Special Agar Noble, Difco Laboratories, Mich., USA) in 0.01 M potassium phosphate buffer (pH 7.2 to 7.5) containing 0.01% Merzonin (thimerosal, Takeda Chemical Industries, Osaka) were poured onto a glass plate (47 × 47 mm) and allowed to solidify as a layer of about 3 mm thickness. Wells for antigens and antisera were cut out as illustrated in the accompanying figures. Some of the antigens used diffused very rapidly into the agar gel, so antiserum was allowed to diffuse from its well overnight before addition of antigens. Plates were placed in an airtight chamber with moistened filter paper and kept at room temperature or at 37 C. Plates were frequently examined for several days for precipitin lines.

7. *Microscopy*

An aliquot of the reaction mixture for protoplast production was centrifuged at 11,000 × *g* for 30 min. The precipitate was resuspended in an appropriate amount of 0.75 M sucrose containing 10% (v/v) formalin and the suspension was kept at room temperature for a few days. The fixed "cells" were thoroughly rinsed with deionized water and used for optical or electron microscopy. For the latter purpose, the "cells" were mounted on a formval film made on a wire mesh and shadowed with platinum/palladium and carbon (Oken Shoji Co., Tokyo) at an angle of 15 degrees to the plane of the film. The shadowed specimens were examined with a Japan Electron Optics Laboratory electron microscope, Model JEM-7. The cytoplasmic membrane fraction was examined with the electron microscope in a similar way but without formalin fixation.

RESULTS

1. *Optimal conditions for production of protoplasts using the L-11 enzyme*

Production of protoplasts of *S. aureus* with the staphylolytic enzymes was first demonstrated in our laboratory on strain Newman 1 using the L-11 enzyme (Kato et al., 1960). However, the osmotically fragile spherical bodies produced were not examined to see if they were completely devoid of cell wall materials. Moreover, subsequent studies showed that the successful production of protoplasts depends on various experimental conditions. Therefore, the influences of the concentration of sucrose, as stabilizer, the age of the test organisms, the pH of the reaction system and the duration of incubation upon the production of stable protoplasts were analyzed to determine the optimum conditions for protoplast production.

Reaction mixtures (3.2 ml) consisted of 0.8 ml of cell suspensions (optical density at 550 mμ, OD_{550}, 2.0 to 2.4) of various ages, 0.4 ml of 0.08 M potassium phosphate buffers of various pH values, 1.6 ml of sucrose solutions of different concentrations, 0.1 ml of L-11 enzyme solution containing 1200 whole cell lytic units/ml (at pH 8.0) and 0.3 ml of deionized water. The reaction mixtures (system ES) were incubated at 37 C for various lengths of time with three control systems: one without sucrose (system E), one without enzyme (system S) and the third without either sucrose or enzyme (system N) (see Table 1). To see whether stable protoplasts were produced, the change of OD_{550} in the reaction mixture, release of material with maximum absorption

TABLE 1. *Composition of the test and control reaction mixtures*

Ingredient	Volume (ml) ES	E	S	N
Cell suspension, OD_{550} 2.0–2.4	0.8	0.8	0.8	0.8
L-11 enzyme, 1200 whole cell lytic units/ml	0.1	0.1	—	—
Sucrose, 0.5–2.0 M	1.6	—	1.6	—
0.08 M potassium phosphate buffer, pH 6.5–8.0	0.4	0.4	0.4	0.4
Deionized water	0.3	1.9	0.4	2.0

at 260 mμ (referred to as the UV-absorbing materials hereafter) into the supernatant fluid, susceptibility of the " cells " to osmotic shock, and morphological changes revealed by gram stain in the test and control reaction systems were followed.

Sucrose concentration: Cells in the middle of the stationary phase (16 hr old culture) were incubated with the L-11 enzyme at pH 7.2 in the absence and presence of various concentrations of sucrose, and the reduction in optical density of the reaction mixture and release of UV-absorbing materials from the " cells " were determined. In this and the following experiments, ultraviolet absorption measurements were performed on the supernatant fluids obtained by centrifugation of the reaction mixtures at 12,000×*g* for 30 min. The results in Fig. 1 show that the presence of sucrose at a final concentration of 0.75 to 1.0 M in the reaction mixture stabilizes the " cells " or protoplasts produced.

Age of test organisms: Protoplast formation from cells in the early, mid and late stages of logarithmic growth and the early, mid and late stages of the stationary phase were examined at pH 7.2 and in the presence of 0.75 M sucrose. Fig. 2 shows that cells harvested in the early or mid stage of the stationary phase (8 hr or 16 hr old culture) gave the most satisfactory results. The suspension of cells in the late stationary phase showed progressive reduction in optical density on incubation for 2 hr, and cells in the logarithmic phase released a considerable amount of UV-absorbing materials during incubation, although reduction in OD_{550} of their suspensions did not exceed 60% of the initial value.

ph of the reaction mixture: The influence of the pH of the reaction mixture on the production of stable protoplasts from cells in the early part of the stationary phase in the presence of 0.75 M sucrose is shown in Fig. 3. When the osmotic fragility of " cells " obtained by centrifugation (at 12,000×*g* for 30 min) of the reaction mixtures were examined by resuspending them in their original volume of deionized water, bursting of " cells " was incomplete at pH values below 7.0, presumably owing to insufficient degradation of cell wall peptidoglycan. At pH values above 7.5, on the other hand, there was considerable libe-

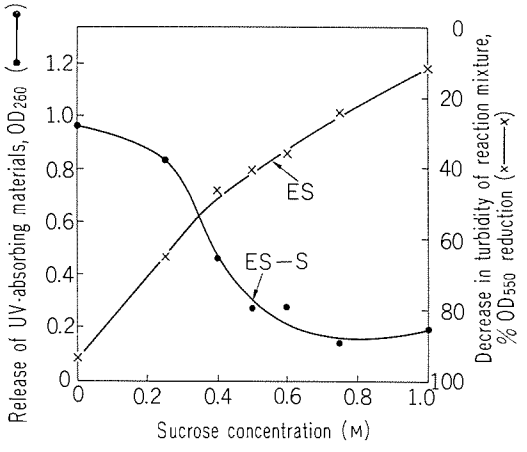

FIGURE 1. *Effect of sucrose concentration in the reaction mixture in protection of protoplasts from osmotic lysis.*
Test organisms: cells in the mid stationary phase
pH of the reaction mixture: 7.2
ES-S: the differences between ES and S

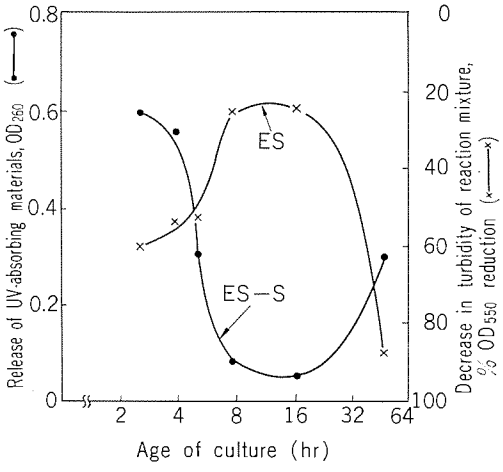

FIGURE 2. *Effect of age of culture on protoplast production.*
Sucrose concentration: 0.75 M
pH of the reaction mixture: 7.2
ES-S: the differences between ES and S

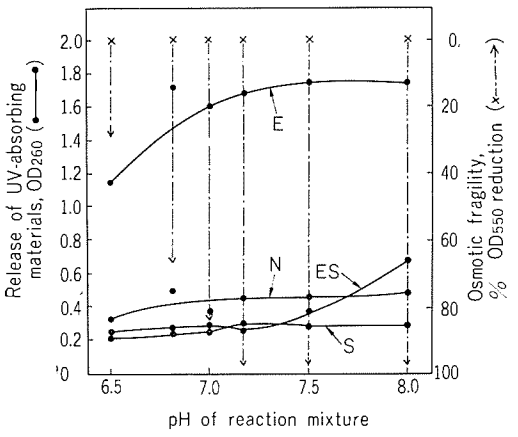

FIGURE 3. *Effect of pH of reaction mixture on protoplast production.*
Test organisms: cells in the early stationary phase
Sucrose concentration: 0.75 M

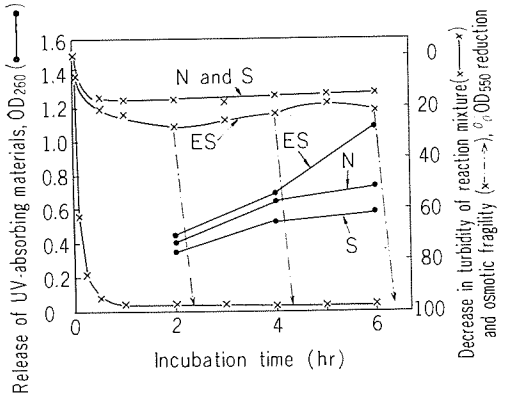

FIGURE 4. *Release of the UV-absorbing materials from protoplasts during prolonged incubation.*
Test organisms: cells in the early stationary phase
Sucrose concentration: 0.75 M
pH of the reaction mixture: 7.2

ration of UV-absorbing materials from the "cells", and the "cells" were osmotically fragile even in the presence of 0.75 M sucrose. Thus, the optimum pH of the reaction mixture was 7.2, although the activity of the L-11 enzyme was not maximal at this pH.

Length of reaction period: Cells in the early stationary phase were incubated with the L-11 enzyme in the presence of 0.75M sucrose at pH 7.2. The OD_{550} reduction, liberation of the UV-absorbing materials and extent of osmotic bursting were examined at intervals during incubation for 6 hr. Fig. 4 shows that the OD_{550} of the test reaction mixture (system ES) decreased rapidly at first and then showed no further decrease during further incubation, while there was significant release of UV-absorbing materials on incubation for 2 hr or longer. An appreciable liberation of UV-absorbing materials was also noticed in the control systems (N and S) containing no enzyme on prolonged incubation. The development of osmotic sensitivity of the "cells" in system ES was complete after a two hr incubation as indicated by complete bursting.

2. *Preparation of protoplasts under standard experimental conditions*

A portion of cells (3.4 g dry weight) of FDA 209P from an 8 hr old culture was washed with cold deionized water, and suspended homogeneously in 100 ml of deionized water to give an OD_{550} of about 44. The cell suspension was poured into a solution, prewarmed to 37 C,

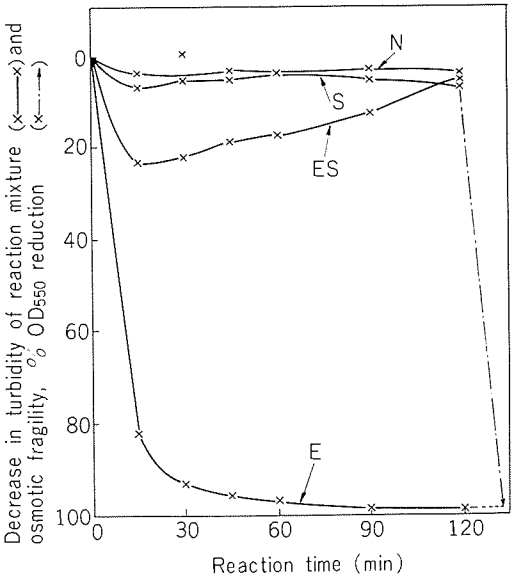

FIGURE 5. *Production of protoplasts of S. aureus FDA 209P with the L-11 enzyme in the presence of 0.75 M sucrose at pH 7.2.*

consisting of 250 ml of L-11 enzyme solution containing 300,000 whole cell lytic units, (at pH 8.0) 400 ml of 1.5 M sucrose and 50 ml of 0.16 M potassium phosphate buffer, pH 7.2. This reaction mixture (system ES) was incubated at 37 C with continuous and gentle mixing with a magnetic stirrer. Controls (systems E, S and N, 9.6 ml each) without sucrose, enzyme or both were also set up and incubated similarly.

At intervals, aliquots (250 μl) were withdrawn and diluted 10-times with 0.75 M sucrose (systems ES and S) or deionized water (systems N and E) to follow changes in the OD_{550} (Fig. 5). In the absence of sucrose (system E), the cell suspension underwent a rapid and almost complete loss of turbidity due to the action of the L-11 enzyme, while in the presence of sucrose (system ES) there was only an initial reduction in the turbidity (about 25% of the starting value) in the first 15 min, and then the OD^{550} of the reaction mixture tended to increase rather than decrease.

Microscopic observations on specimens withdrawn at intervals, fixed with formalin in 0.75 M sucrose and stained with gram-stain revealed that control cells with intact cell walls (from systems N and S) occurred in packet or clusters as gram-positive spheres, while " cells " incubated with the L-11 enzyme in the presence of 0.75 M sucrose (from system ES) were present as scattered, single gram-negative spherical bodies (Fig. 6). After incubation for 2 hr, an aliquot of system ES was centrifuged at 25.000 $\times g$ for 30 min. The pellet was fixed and examined electron-microscopically. The photomicrograph in Fig. 7 shows, electron-dense, highly shrunken spheres representing protoplasts and membraneous structures presumably produced by bursting of protoplasts during fixation.

Another aliquot of the test reaction mixture (system ES) was centrifuged at 25,000 $\times g$ for 30 min, and the " cell " pellet was washed once with 0.75 M sucrose. When the pellet was then resuspended in the original volume of 0.005 M magnesium sulfate solution, the " cells " were lyzed almost instantaneously. The ultraviolet absorption spectra of the supernatant fluid of system ES, the sus-

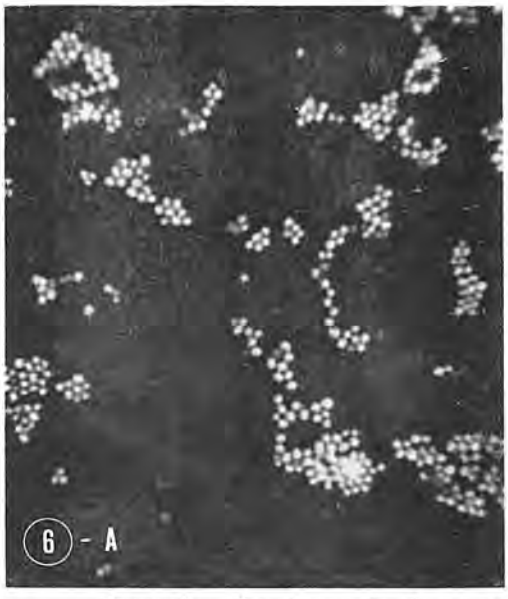

FIGURE 6. *Photomicrographs of gram-stained pellets from system S (A) and system ES (B).*

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FIGURE 7. *Electron micrograph of the deposit from system ES, fixed with 10% formalin in 0.75 M sucrose. Shadow-cast with platinum/palladium and carbon. The bar indicates 0.3 μ.*

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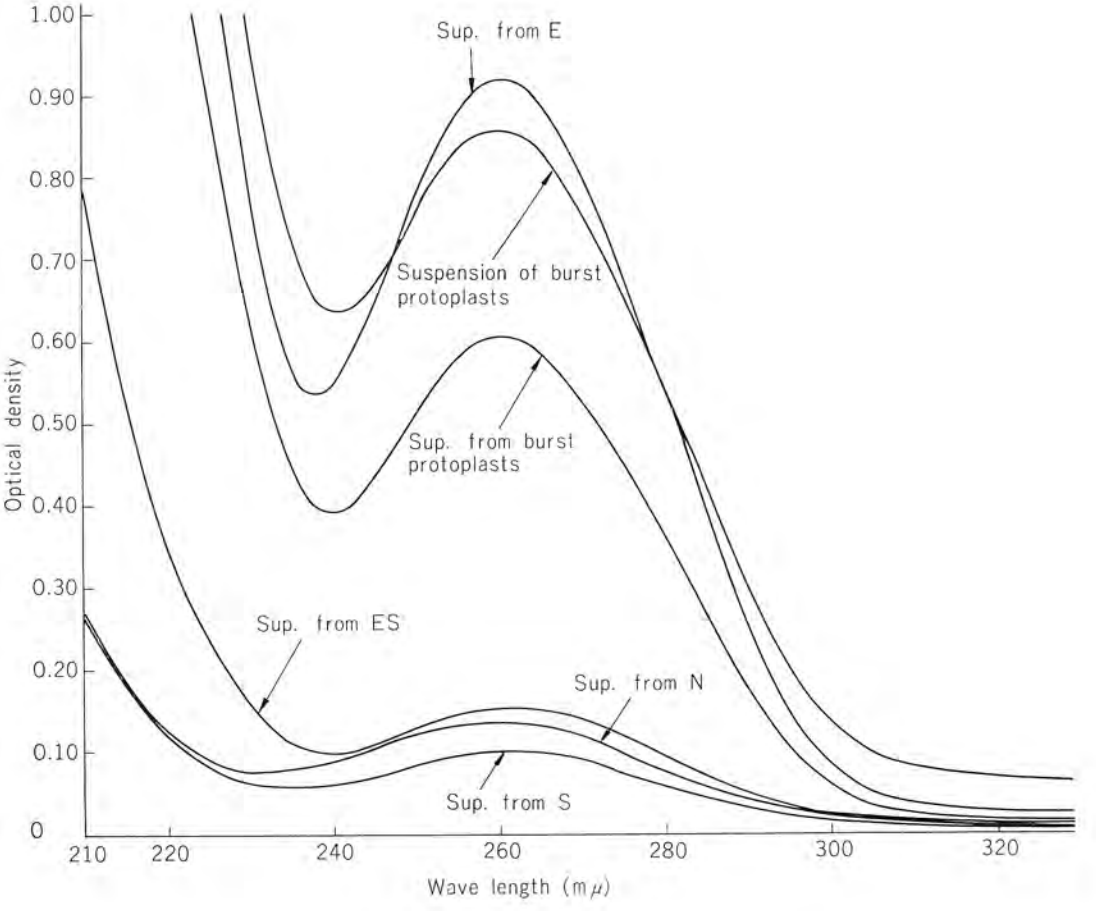
FIGURE 8. *Ultraviolet absorption spectra of the supernatant fluids from test and control reaction mixtures (systems ES, E, S and N), a suspension of burst protoplasts and the supernatant fluid from burst protoplasts.*



pension of burst " cells " and the supernatant fluid of the burst " cell " suspension (obtained by centrifugation at 25,000×*g* for 30 min) and the supernatant fluids from control reaction mixtures (systems N, E and S) were compared. Fig. 8 shows that there were no significant differences between the ultraviolet absorption spectra in the range of 240 mμ to 290 mμ of the supernatant fluids from systems ES, S and N. Thus there was no appreciable lysis of " cells " during dissolution of their cell walls by the L-11 enzyme in the presence of 0.75 M sucrose. A large quantity of UV-absorbing materials was liberated when the " cells " deposited from the system ES were suspended in dilute magnesium sulfate solution. The intensity of absorption at 260 mμ shown by suspension of burst " cells " was similar to that of the supernatant fluid from system E containing enzyme but no stabilizer. Thus the " cells " from system ES were completely osmotically fragile. A considerable amount of UV-absorbing materials in the suspension of burst " cells " was deposited together with the ghosts on centrifugation at 25,000×*g* for 30 min.

3. *Isolation of cytoplasmic membranes from burst " cells "*

Test reaction mixture (system ES) was incubated for 2 hr and then centrifuged at 25,000×*g* for 30 min. The osmotically sensitive " cells " (protoplasts) precipitated were washed once with 430 ml of 0.75 M sucrose and suspended in 770 ml of 0.005 M magnesium sulfate solution. The suspension was stirred until the protoplasts had completely burst. The suspension of burst protoplasts was centrifuged at 37,000×*g* for 30 min and the cytoplasmic membrane fraction deposited was washed 3 times with 100 ml portions of 0.005 M magnesium sulfate solution. The washed fraction was then digested at 37 C for 5 hr with 300 μg of crystalline deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo., USA) in 100 ml of 0.0125 M potassium phosphate buffer, pH 7.4, containing 0.00125 M magnesium sulfate. The digested material was washed once with 65 ml of the same buffer without magnesium sulfate, and treated (at 37 C for 5 hr) with 300 μg of crystalline ribonuclease (Sigma) in 40 ml of the buffer. Then the cytoplasmic membranes were collected by centrifugation at 37,000×*g* for 30 min, and suspended in 20 ml of deionized water. The suspension was centrifuged at 3,000×*g* for 10 min to remove coarse debris. The debris was washed twice with 20 ml portions of deionized water with low speed centrifugation. The supernatant and washing fluids were combined and centrifuged at 37,000×*g* for 30 min to collect purified cytoplasmic membranes. The membranes were thoroughly washed with deionized water and lyophilized. An electron micrograph of this cytoplasmic membrane fraction is shown in Fig. 9. The supernatant fluid of the reaction mixture and that from the burst protoplasts were dialyzed separately at 4 C against large quantities of deionized water. Then they were concentrated under reduced pressure at room temperature with a rotatory evaporator, and lyophilized. The products were designated as the solubilized cell wall and cytoplasmic fractions, respectively. An outline of the procedures for separation of the three fractions is presented in Fig. 10.

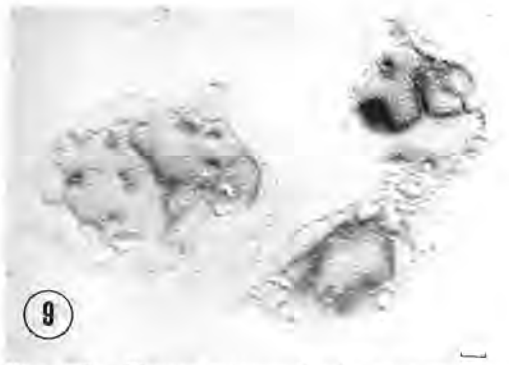


FIGURE 9. *Electron micrograph of the isolated cytoplasmic membrane fraction.*
Shadow-cast with platinum/palladium and carbon. The bar indicates 0.16 μ.

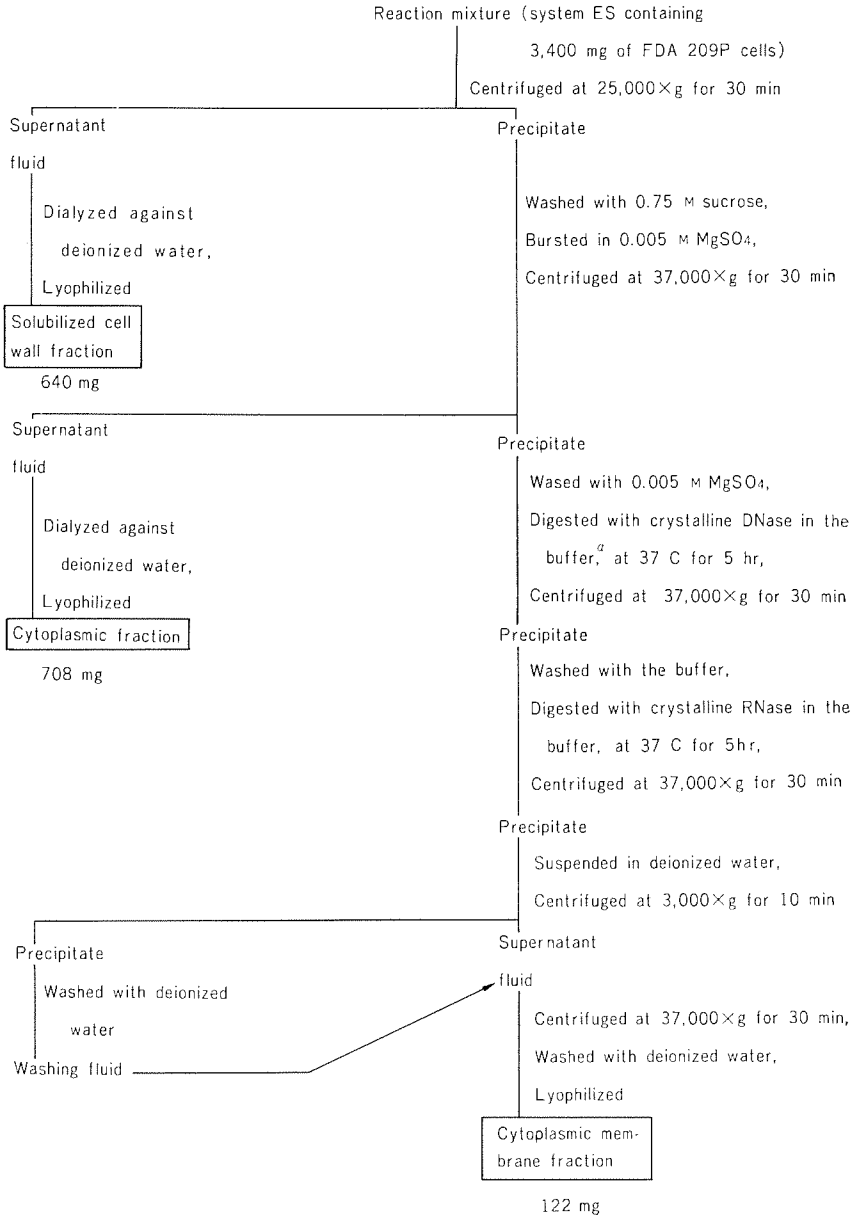


FIGURE 10. *Procedures for separation of the cytoplasmic membrane, solubilized cell wall and cytoplasmic fractions.*

Analytical data on some constituents of the cytoplasmic membranes and the other two fractions are given in Table 2 with the yields of each fraction. The membrane fraction contained negligible amounts of hexosamines and D-amino acids, characteristic components of cell wall peptidoglycans, indicating that no significant amounts of cell wall peptidoglycans remained associated with this fraction. The amino acid and amino suger compositions of the cytoplasmic membrane and solubilized cell wall fractions are given in Table 3. The membrane fraction is virtually free from muramic acid and glucosamine, essential components of the cell wall peptidoglycan. The amino acid content of the membrane fraction was unexpectedly low, in view of the fact that it is known that cytoplasmic membrane preparations from various bacterial species contain about 50 to 75% protein (Salton, 1967). The significance of this finding is discussed later. The solubilized cell wall fraction, on

TABLE 2. *Contents of hexosamines, total phosphorus and D-amino acids and yield of the cytoplasmic membrane, solubilized cell wall and cytoplasmic fractions*

Material	Yield (mg)	Hexosamines A[a]	Hexosamines B[b]	D-amino acids A	D-amino acids B	Total P A	Total P B
Whole cells	3,400	260	884	250	800	760	2,640
Solubilized cell walls (dialyzed)	640	1,225	784	375	240	833	553
Cytoplasmic membranes	122	9	1	58	7	1,050	128
Cytoplasm (dialyzed)	708	14	96	40	28	1,250	885

a mμmoles/mg. *b* total μmoles.

TABLE 3. *Amino acid and amino sugar compositions of the cytoplasmic membrane and solubilized cell well fractions*

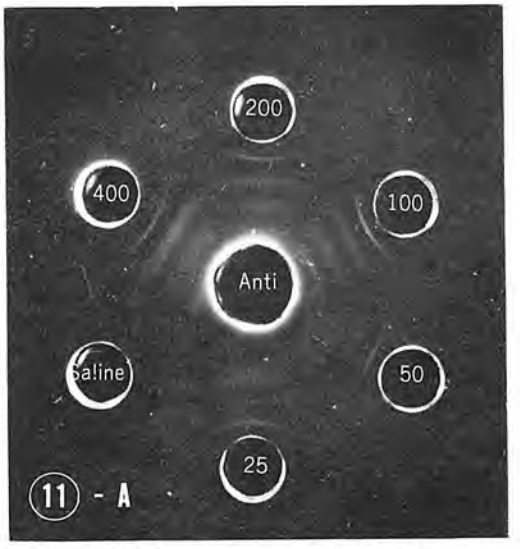
Amino acid and amino sugar	Cytoplasmic membranes mμmoles/mg	Cytoplasmic membranes μg/mg	Solubilized cell walls (dialyzed) mμmoles/mg	Solubilized cell walls (dialyzed) μg/mg
Ala	319	28.4	347	30.9
Lys	175	25.6	79	11.5
Gly	109	8.2	80	6.0
Asp	100	13.3	49	6.5
Leu	93	12.2	31	4.1
Glu (+Pro)	79	11.6[a]	68	10.0[a]
Ser	73	7.7	59	6.2
Phe	64	10.6	—	—
Thr	46	5.5	29	3.5
Ile	44	5.8	(12)	(1.6)
Val	41	4.8	(16)	(1.9)
Arg	38	6.6	—	—
Met	(15)	(2.2)	(5)	(0.7)
His	(14)	(2.2)	—	—
Tyr	(13)	(2.4)	—	—
GlcN[b]	(9)	(1.6)	794	142.3
MurN[c]	—	—	314	78.9
Total		148.6		304.1

a Calculated as glutamic acid. *b* glucosamine. *c* muramic acid.
Values in parenthesis are not reliable.

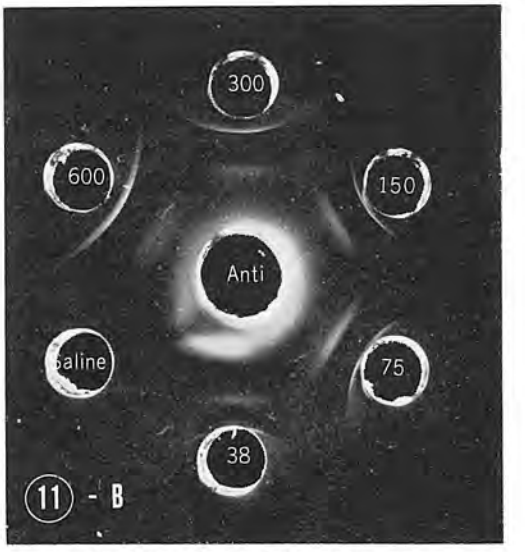
the other hand, had high contents of glucosamine and muramic acid, but contained few amino acids except alanine. This observation is compatible with the established fact that the the L-11 enzyme solubilized *S. aureus* cell walls due to the actions of muramyl-L-alanine amidase and D-alanyl-glycine- and glycyl-glycine-endopeptidases (Kato and Strominger, 1968; Kato et al., 1968). Thus the peptide portion of the cell walls was probably degraded and lost by dialysis, while components belonging to the glycan and teichoic acid portions were retained in the non-dialyzable fraction.

4. *Comparison of the antigens in the cytoplasmic membrane fraction, and the solubilized cell wall and cytoplasmic fractions*

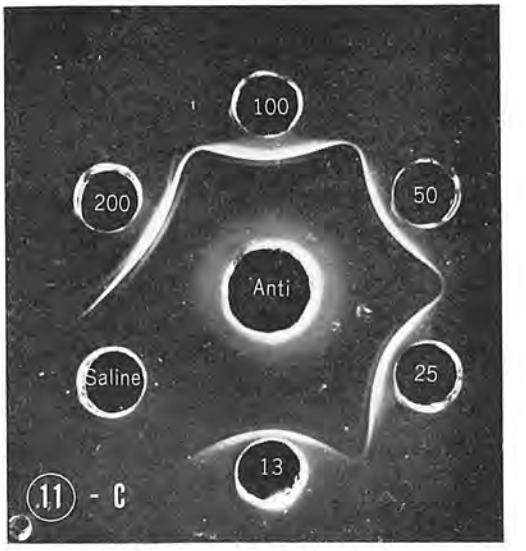
A specimen (16 mg) of the cytoplasmic membranes isolated was suspended in 1 ml of 0.01 M potassium phosphate buffer, pH 7.4 and mixed with 10 μl of 3 N sodium hydroxide. The suspension was submitted to sonic vibration in a Kubota 10 kc/s sonic oscillator (Model KMS-100, Kubota Seisakusho Co., Tokyo) for 20 min. The resulting opalescent "solution" containing a small amount of insoluble residue was neutralized by addition



A: Cyloplasmic membane fraction



B: Solubilized cell wall fraction



C: Cyloplasmic fraction

FIGURE 11. *Immunodiffusion reaction of anti-FDA 209P serum with serial two-fold dilutions of the cytoplasmic membrane, solubilized cell wall and cytoplasmic fractions.*

Figures represent the weight (μg/40 μl) of test fractions in the antigen wells.

Anti: anti-FDA 209P whole cell rabbit serum (70 μl)

of 10 μl of 3 N hydrochloric acid and used as an antigen for immunodiffusion without centrifugation. The solublilized cell wall and cytoplasmic fractions, and specimens of teichoic acid and protein A were dissolved in 0.15 M sodium chloride solution at appropriate concentrations for immunodiffusion.

Fig. 11 shows the reaction of serial two-fold dilutions of the cytoplasmic membrane fraction, and solubilized cell wall and cytoplasmic fractions with rabbit antiserum prepared against whole cells of FDA 209P (the highest concentrations used were 10, 15 and 5 mg/ml, respectively). The membrane fraction gave one sharp and 3 broad precipitation lines. For convenience, these lines will be referred to as k, l, m and n in order from the antigen well to the antiserum well. The solubilized cell wall fraction produced one sharp and 2 or 3 broad lines, and the cytoplasmic fraction gave 3 distinct and 2 faint lines.

The relationships of the lines given by the three test fractions with those of teichoic acid and protein A were studied, as illustrated in Fig. 12. The line closest to the antiserum well given by the solubilized cell wall fraction and the line m of the cytoplasmic membranes fused with the precipitation line of teichoic acid. It is evident that the cells of FDA 209P contained protein A, since anti-FDA 209P rabbit serum reacted with protein A, producing one distinct and one faint precipitation line. However, none of the test fractions produced a line fusing with the protein A line(s).

The cross reactions between the cytoplasmic membrane fraction and solubilized cell wall

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FIGURE 12. *Analysis by immunodiffusion of the antigenic relationships of the cytoplasmic membrane, solubilized cell wall and cytoplasmic fractions with teichoic acid and protein A.*

CM: cytoplasmic membrane fraction, CW: solubilized cell wall fraction, CP: cytoplasmic fraction, TA: teichoic acid, PA: protein A, Anti: anti-FDA 209P whole cell rabbit serum (70 μl)

Figures in parenthesis represent the weight (μg/40 μl) of test materials in the antigen wells.

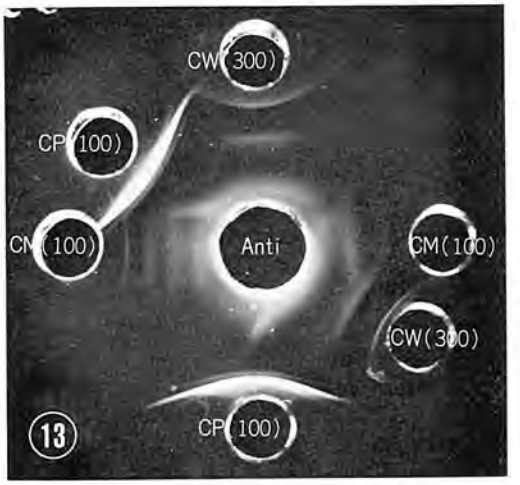

FIGURE 13. *Analysis by immunodiffusion of the identity or non-identity of the precipitation lines given by the cytoplasmic membrane, solubilized cell wall and cytoplasmic fractions.*

CM: cytoplasmic membrane fraction, CW: solubilized cell wall fraction, CP: cytoplasmic fraction, Anti: anti-FDA 209P whole cell rabbit serum (70 μl)

Figures in parenthesis represent the weight (μg/40 μl) of test fractions in the antigen wells

and cytoplasmic fractions are shown in Figs. 13 and 14. The precipitation pattern of the membrane fraction shown in Fig. 14 was somewhat different from those in Figs. 11 and 13. The line k was faint, and the line 1 was separated into two lines (l_1 and l_2). Comparison of the precipitation patterns given by each of the test fractions and that of teichoic acid reveals that the line m of the membrane fraction, the line closest to the antiserum well of the solubilized cell wall fraction and one of the lines produced by the cytoplasmic fraction fused with the line given by teichoic acid. The line n of the

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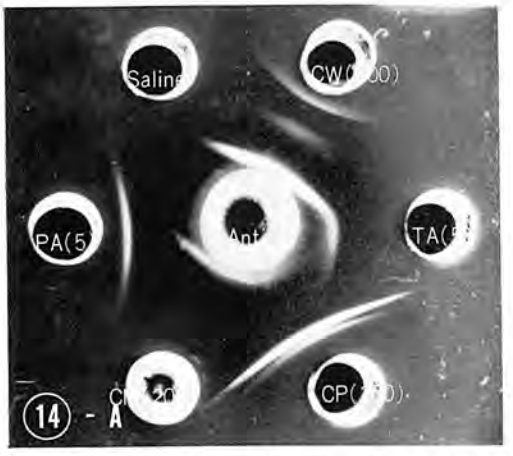
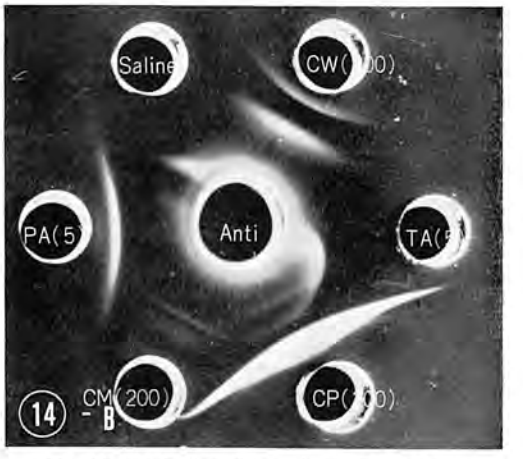
FIGURE 14. *Analysis by immunodiffusion of the identity or non-identity of the precipitation lines given by the cytoplasmic membrane, solubilized cell wall and cytoplasmic fractions, teichoic acid and protein A.*

CM: cytoplasmic membrane fraction, CW: solubilized cell wall fraction, CP: cytoplasmic fraction, TA: teichoic acid, PA: protein A, Anti: anti-FDA 209P whole cell rabbit serum (70 μl)

Figures in parenthesis represent the weight (μg/40 μl) of test materials in the antigen wells.



A : Precipitation lines after 18 hr's reaction at room tempreature



B : Precipitation lines after 72 hr's reaction at room temperature

membrane fraction which moved rapidly in agar gel, fused with the line closest to the antiserum well of the cytoplasmic fraction. The presence of an antigen related to the antigens of the cytoplasm in the solubilized cell wall fraction was indicated by fusion with spur formation of the second line from the antigen well of the cytoplasmic fraction with the line nearest to the antigen well of the solubilized cell walls. The line k was found to have a peculiar feature. Some batches of antigen " solution " of the cytoplasmic membranes did not produce this line at the concentration examined, while another batches gave this line on reaction not only with homologous anti-FDA 209P serum, but also with preimmune rabbit sera as well as antisera against bacteria other than *S. aureus*. These findings indicate that line k may not be the result of a true antigen-antibody reaction.

The sensitivities to heat and trypsin of the antigens responsible to the precipitation lines given by the cytoplasmic membranes were studied. It was found that the precipitation pattern was not changed by either heating at 100 C for 10 min or by digestion with crystalline trypsin (Trypsillin, Mochida Pharmaceutical Co., Tokyo). Digestion was performed by incubating a 100 μl aliquot of the membrane " solution " with 50 μg of crystalline trypsin in 0.01 M potassium phosphate buffer, pH 7.4, for 3 hr at 37 C.

DISCUSSION

Mitchell and Moyle (1957) first described the " protoplasts " of *S. aureus* and demonstrated that when *S. aureus* strain Duncan in the logarithmic phase of growth was incubated at 25 C in 1.2 M sucrose at pH 5.8, the organisms lost their rigid and tensile cell walls by an autolytic process and released spheres which were stable in 1.2 M sucrose but lyzed in media of lower osmotic pressure. Since then, protoplasts or spheroplasts of *S. aureus* have been produced by several investigators using staphylolytic enzymes of microbial origin: strain Newman 1 with *Flavobacterium* L-11 enzyme (Kato et al., 1960), strain H with *Chalaropsis* endo-*N*-acetylhexosaminidase (Hash, Wishnick and Miller, 1964), strain FDA 209P with lysostaphin (Schuhardt and Klesius, 1968, Schuhardt, Huber and Pope, 1969; Huber and Schuhardt, 1970) and strains H and 100 with a lytic enzyme from *Streptomyces griseus* (Ward and Perkins, 1968). However, the immunological properties of the surface membranes of the protoplasts or spheroplasts obtained were not investigated by these workers.

The antigens of the cytoplsmic membranes of *S. aureus* were first studied by Freimer (1963) during extensive studies on the immunological properties of group A streptococcal cytoplasmic membranes, to clarify the immunological relationship between the membranes of group A streptococci and *S. aureus* strain NYH 6. It was shown by immunodiffusion that trypsin extracts of *S. aureus* membranes, isolated by mechanical disruption of the cells and differential centrifugation of the disrupted cell suspension, gave a single precipitation line with rabbit antiserum against staphylococcal membranes, and the antigen of *S. aureus* membranes was unrelated to the antigens present in the membranes of Group A *S. pyogenes*.

The present study showed that the cytoplasmic membranes of *S. aureus* FDA 209P isolated from protoplasts prepared using the L-11 enzyme were virtually free from components of cell wall peptidoglycans, and produced 4 or 5 precipitation lines on immunodiffusion with rabbit antiserum against the whole cells of the homologous organism. Of these precipitation lines, the line k was probably produced by a non-immunological reaction between a nonantigenic component of the membranes and normal serum protein. A similar non-immunological reaction of protein A, the outermost component of *S. aureus* cell walls, with sera of many mammalian species was extensively studied by Sjöquist and his coworkers (Sjöquist and Stälenheim, 1969) and other (Nickerson et al., 1970). However,

the line k was not due to protein A contaminating the membrane fraction since there was no cross reaction between the line k and the lines given by a specimen of protein A. Line m, on the other hand, was found to be produced by teichoic acid which had been solubilized by degradation of cell wall peptidoglycans and then attached on the membranes. Of the remaining precipitation lines produced by the cytoplasmic membrane fraction, lines l_1 and l_2 almost certainly represented the reaction of the antigens proper to the cytoplasmic membranes and not shared by either cell walls or cytoplasm. However, the antigen forming the line n may not be specific to the membranes, since this line fused with one of the lines produced by the cytoplasmic fraction.

It seems probable that the cytoplasmic membranes of *S. aureus* actually contain more antigens than those found in the present study. One possibility is that the membrane antigens with a protein nature may be lost during production of protoplasts with the L-11 enzyme, since a partially purified preparation of the L-11 enzyme was found to have casein-digesting activity (Hirata, 1970). This suggestion seems to be supported by the observation (Hirachi, Kotani and Harada, 1970) that sonicated preparations of *M. lysodeikticus* cytoplasmic membranes isolated using crystalline egg white lysozyme gave 7 to 8 precipitation lines on reaction with antisera to the membrane fraction, and that the antigens responsible for 3 of the lines were destroyed by treatment with the L-11 enzyme preparation or with crystalline trypsin or pronase. In this connection, it is noteworthy that none of the membrane antigens of *S. aureus* were susceptible to crystalline trypsin. Another possibility is that antisera prepared against the cytoplasmic membranes of *S. aureus* may reveal the existence of larger number of membrane specific antigens, since some of them may have low immunogenicity. Our results with the cytoplasmic membranes of *M. lysodeikticus* indicates that this is the case.

One of the the technical problems encountered in antigen analysis of biological membranes, including bacterial cytoplasmic membranes, is how to solubilize the antigenic components incorporated into the membranes as building blocks. In the present study, the isolated cytoplasmic membrane fraction was "solubilized" by sonic vibration at an alkaline pH. The resulting "solution" was suitable as antigen solution in immunodiffusion. It should be pointed out, however, that the "solution" prepared by sonication consists of minute particles resulting from random, physical fragmentation of cytoplsmic membranes, and it is not a true solution of membrane components. Therefore, sonic disruption of cytoplasmic membranes may produce particles with the same antigenic determinants but different diffusion coefficients in agar gel, though the formation of particles with fairly uniform sedimentation coefficients by sonication of bacterial membranes has been reported by Salton and Netschey (1965) and Brown (1965). This should be taken into consideration for a proper interpretation of the results of the immunodiffusion obtained using an antigen "solution" of this kind.

There are significant differences in the amino acid contents and compositions of the membrane fraction analyzed in this study and of the membranes from protoplasts and L-forms of strains H and 100 isolated and analyzed by Ward and Perkins (1968). These differences may be because the present membrane fraction had lost a considerable portion of protein components by digestion with the protease(s) in the L-11 enzyme preparation. The fact that the protoplasts produced with the L-11 enzyme were quite stable although considerable amounts of protein components had been removed from their surface membranes indicates that the present analytical results may reflect the amino acid content and composition of the structural proteins of cytoplasmic membranes of *S. aureus* better than those reported by Ward and Perkins.

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