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SOLUBILIZATION OF PHAGE RECEPTOR SUBSTANCES FROM CELL WALLS OF *STAPHYLOCOCCUS AUREUS* (STRAIN COPENHAGEN) BY CELL WALL LYTIC ENZYMES[1]

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SUMMARY Phage receptor substances which irreversibly inhibit phage 3C were solubilized and isolated from the cell walls of *Staphylococcus aureus* strain Copenhagen (phage type 3C/3B/55+) using the pentaglycine bridge splitting enzyme of *Flavobacterium* L-11 bacterium and the Chalaropsis (B) enzyme. There was no phage inhibiting activity in a purified teichoic acid fraction obtained by cold trichloroacetic acid extraction of the cell walls.

Chemical analyses showed that the receptor substance (BS-B fraction) isolated by gel filtration and ECTEOLA-cellulose column chromatography from the cell walls solubilized by the bridge splitting enzyme was a complex of glycopeptide interconnected in a glycan portion (with an average chain length of about 4 disaccharide units) and teichoic acid. The active substance (Chala-B fraction) obtained from a non-dialysable portion of the Chalaropsis enzyme digest of the walls by ECTEOLA-cellulose column chromatography was a complex of teichoic acid and a polymer of several disaccharide-peptide subunits interconnected by pentaglycine cross bridges.

Hydrolysis of the linkages between the glycan and the peptide moieties in these receptor substances with the L-11 enzyme containing *N*-acetylmuramyl-L-alanine amidase was accompanied by a marked reduction in the inhibitory activity toward phage 3C. On the other hand, either treatment of the BS-B fraction with the Chalaropsis enzyme or hydrolysis of the Chala-B fraction with the L-11 bridge splitting enzyme resulted in almost complete depolymerization of the glycopeptide portion of these fractions, but did not cause any significant loss of the phage 3C inhibiting activity.

It was concluded from these studies that all three main components of *S. aureus* cell walls, teichoic acid, glycan and peptide moieties, must be linked together into a complex to exhibit an inhibitory effect against phage 3C, but polymerization of the complex through glycosidic linkages in the glycan portion or pentaglycine bridges in the peptide portion was not necessary for manifestation of the phage 3C receptor

1 A part of this work was read at the 39th Annual Meeting of the Japan Microbiological Society (at Hiroshima, in April, 1966) and at the 41st Annual Meeting of the same Society (at Tokyo, in April, 1968).

activity.

No inhibitory activity was observed in either the BS-B or Chala-B fraction against other test phages, including phage 3B capable of lysing cells of strain Copenhagen.

INTRODUCTION

One approach to the problem of the chemical nature of the bacterial receptor sites for phage adsorption involves isolation of the receptor substances from the bacterial cells and their chemical characterization.

Several investigations along these lines on phage receptor sites of *Staphylococcus aureus* have been made since the first demonstration of the phage inactivating capacity of extracts from autolyzed staphylococci by RAKIETEN, RAKIETEN and DOFF (1936). With the development of techniques for isolation of a satisfactorily intact and pure cell wall fraction from mechanically disrupted bacterial cells (SALTON and HORNE, 1951, and others), it was demonstrated by HOTCHIN, DAWSON and ELFORD (1952) that phage receptor activity toward phage K was exclusively localized in the particulate cell wall fraction of strain Oxford and was not solubilized on disruption of the cells. This observation was confirmed and extended by MORSE (1962), who demonstrated that the cell walls isolated from *S. aureus* strain NYH-6 inactivated a variety of different staphylococcal bactriophages, irrespective of the ability of the test phages to lyse the cells of strain NYH-6. He failed, however, to demonstrate phage receptor activity in either a mucopeptide (peptidoglycan) or a teichoic acid fraction. Similar results were obtained by MATTHEW and ROSENBLUM (1967). ROSATO and CAMERON (1964), on the other hand, isolated a particulate phage receptor site for phage 77 from the cell walls of strain 77 by vigorous sonic oscillation and density-gradient centrifugation. They proposed that this substance contained no organic phosphates and was a mucopeptide devoid of teichoic acid. This observation was followed by a recent study of CAMERON and OSBORNE (1967) showing that the mucopeptide precursor, *N*-acetylglucosaminyl-*N*-acetylmuramyl-peptide phospholipid could inactivate phage 77.

In contrast to the studies of CAMERON and his colleagues, WOLIN, ARCHIBALD and BADDILEY (1966) presented evidence suggesting that differences in phage susceptibility among *S. aureus* mutants was parallel to chemical differences in the teichoic acid moieties. In agreement with this, it was demonstrated by COYETTE and GHUYSEN (1968) that substitution of 4-*O*-β-(*N*-acetyl-D-glucosaminyl) on the D-ribitol units of the teichoic acid moiety in a definite configuration was essential for fixation by cell walls of *S. aureus* strain Copenhagen of several phages selected on the basis of differences in type series, serological groups, morphology and infectivity.

None of the above workers succeeded in actual solubilization of phage receptor sites from *S. aureus* walls. Further, there are definite discrepancies between the results reported on the chemical nature of phage receptor substances of *S. aureus*. Thus we attempted to solubilize the phage receptor sites of *S. aureus* strain Copenhagen cell walls using the L-11 enzyme (KOTANI *et al.*, 1959; KATO *et al.*, 1962; KATO and STROMINGER, 1968; KATO *et al.*, 1968) and the Chalaropsis (B) enzyme (HASH, 1962, 1963; TIPPER, STROMINGER and GHUYSEN, 1964; HASH and ROTHLAUF, 1967), and to elucidate the chemical structure responsible for the phage inactivating activity.

MATERIALS AND METHODS

1. *Bacteriophages and their propagating bacteria*

Staphylococcal phages, 3C (NCTC 8411), 3B (NCTC 8410), 42D (NCTC 8414) and 53 (NCTC

8406), and propagating organisms, namely *S. aureus* strains 3C (NCTC 8327), 3B (NCTC 8321), 42D (NCTC 8341) and 53 (NCTC 8511) were kindly supplied by Dr. K. MAESHIMA, Public Health Research Institute of Kobe City, Hyogo.

2. *Culture of S. aureus*

S. aureus strains Copenhagen, Duncan and 3528, in which phage inhibiting activity was examined, and the strains used as propagating organisms for phages, were cultivated with shaking in nutrient broth (pH 7.2) supplemented with 0.1% glucose and 0.5% dried yeast extract (Daigo Nutritional Chemicals, Osaka). All cultures were grown at 37°C for 18 hr.

3. *Preparation of phage stocks*

Phages were propagated in the following way. One hundred ml of nutrient broth supplemented with 0.25% powdered agar (Nissui Seiyaku Co., Tokyo) was inoculated with one ml each of a phage stock and an overnight culture of an appropriate bacterial strain, and incubated at 37°C on a reciprocal shaker. Shaking was continued for 18 hr and then cell debris was removed by centrifugation at 6,500 *g* for 20 min. The supernatant containing phages was mixed with a few small pieces of thymol and stored at 4°C. The titers of phages (plaque forming units, PFU/ml) in the crude lysates obtained were as follows: phage 3C— 8×10^{10}, 3B—10^{9}, 42D—3×10^{8} and 53—10^{7}.

The purified phage 3C stock which was used in most experiments was obtained by centrifugation of phages in a crude phage stock in a Hitachi refrigerated centrifuge (Model 55PA with a RP21A rotor, Hitachi Ltd., Tokyo) at 40,210 *g* for 60 min and by repeated washing of the precipitate with the original volume of 0.01 M phosphate buffer, pH 7.2, until the absorptions of the washings at 260 and 280 mμ became negligible. The purified phage pellet was resuspended in the original volume of buffer. The resulting purified phage suspension (10^{9} PFU/ml) was stored in one ml aliquots at −20°C, avoiding freezing and thawing.

4. *S. aureus cell walls*

Specimens of pronase-treated, or trypsinized cell walls of *S. aureus* strain Copenhagen were prepared as described in previous papers (SUGINAKA *et al.*, 1967; KATO *et al.*, 1968; SUGINAKA *et al.*, 1968). A phage type of strain Copenhagen was shown by the standard procedure of phage typing to be 3C/3B/55+. The pronase-treated cell walls used in most experiments had the following chemical composition (mμmoles/mg, moles/mole of total glutamic acid residues in parenthesis): muramic acid 420 (0.8), glucosamine 710(1.4), alaine 1,010(2.0), glutamic acid 500(1.0), lysine 590(1.2), glycine 2,475(5.0) and organic phosphorus 614(1.2). The amount of alkali-labile D-alanine residues known to be ester-linked to the ribitol units of teichoic acids was only 0.1 mole of glutamic acid.

5. *Cell wall lytic enzymes*

Flavobacterium L-11 bacterium was grown with aeration in 2% glucose broth. A crude enzyme (lot 2) was prepared by precipitation of the culture supernatant with ammonium sulfate.[2] A specimen of the L-11 enzyme (unfractionated) was obtained by gel filtration of this enzyme solution on columns of Sephadex G-75 and G-25 connected in series by polyethylene tubing. This enzyme specimen had *N*-acetylmuramyl-L-alanine amidase activity as well as D-alanyl-glycine-, and glycyl-glycine-endopeptidase activities toward the cell walls of *S. aureus* strain Copenhagen, as reported by KATO and STORMINGER (1968) and KATO *et al.* (1968). This enzyme specimen was further fractionated by Amberlite IRC-50 column chromatography and an enzyme preparation (the L-11 endopeptidase, or the bridge splitting enzyme) which had endopeptidase activities but lacked the amidase activity was separated. The details of the isolation procedures used and characterization of the L-11 endopeptidase will be published elsewhere.

The Chalaropsis (B) enzyme was a generous gift of Dr. P. A. MILLER, (LEDERLE Laboratories, American Cyanamid Co., New York, USA).

6. *Exo-β-N-acetylglucosaminidase*

This enzyme was prepared from a pig epididymis by the method of FINDLAY, LEVVY and MARSH (1958). Five μl of a stock solution of the enzyme specimen obtained was shown to hydrolyze 50 mμ moles of p-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Pierce Chemical Co., Rockford, Ill. USA) completely in 2 hr under the assay conditions defined by the authors, except that buffers were used at one fifth the concentration in the original report. Free *N*-acetyl-

2 This crude enzyme specimen was prepared in the Fuji pilot plant (Mishima, Shizuoka) at the Kyowa Fermentation Industry, Tokyo.

glucosamine residues liberated by hydrolysis were estimated by the method of REISSIG, STROMINGER and LELOIR (1955) with a heating time of 7 min. The stock enzyme solution was dispensed and stored in a deep freezer before use.

7. *Phage inhibition test*

The ability of test specimens to inhibit bacteriophages was tested as follows. The assay system contained a phage suspension in nutrient broth (10^4 PFU/ml), a solution of test specimens in deionized water or deionized water in the control, and nutrient broth or broth supplemented with 0.02 M $CaCl_2$ in the volume ratio of 1 : 1 : 3. This was put into an L-shaped glass tube (internal diameter 1.5 cm and length of horizontal part 15 cm). The tube was incubated with shaking in a water bath at 30°C. After 30 min (or if necessary, 60, 120 or 180 min) incubation, 0.5 ml aliquots were mixed with 0.5 ml of a overnight broth culture of the propagating (indicator) strain of *S. aureus* and 2.5 ml of a soft nutrient agar (agar content, 0.9%) melted and held at about 48°C. The entire contents of the tubes were poured onto 20 ml of basal nutrient agar (agar content, 1.5%) in Petri dishes. Plaques were counted after overnight incubation at 30°C. The phage inhibiting activity of test specimens was expressed as per cent reduction in PFU, which was calculated as follows: 100−(PFU in the test plate/PFU in the control plate without test specimen)× 100. Duplicate assays were performed on each test specimen and the values for per cent reduction in PFU are given as averages.

8. *Analytical methods*

Total, N-terminal and C-terminal amino acids were determined by quantitative thin layer chromatography of the dinitrophenylated derivatives, as described by GHUYSEN, TIPPER and STROMINGER (1966) with minor modifications. Muramic acid and glucosamine along with amino acids were estimated by quantitative paper chromatography by the method of PRIMOSIGH *et al.* (1961).

Total hexosamines were determined by the method of ROSEMAN and DAFFNER (1956) modified by GHUYSEN *et al* (1966). Total and inorganic phosphorus, and reducing sugars were measured as described previously (KATO *et al.*, 1968).

Color intensities in these determinations were estimated in a Hitachi-Perkin-Elmer spectrophotometer (Model 139 UV-VIS, Hitachi Ltd., Tokyo), using micro-cells with a light path of 1 cm and capacity of about 300 μl.

9 *The average chain length of a glycan portion of glycopeptide*

This was calculated by taking the molar ratio of muramic acid residues to formaldehyde liberated by periodate oxidation of a test specimen reduced with sodium borohydride, as the average number of repeating disaccharide units (TIPPER, STROMINGER and ENSIGN, 1967). Aliquots (30 μl) of specimens containing 40–80 mμmoles of muramic acid were reduced with 3 μl of fresh, unbuffered 1 M sodium borohydride solution at room temperature for 5 hr. After neutralization with 3 μl of 1 M acetic acid, the solution was mixed with 45 μl of 0.019 M acetate buffer, pH 4.7 and 10 μl of 0.012 M sodium periodate, and was oxidized at room temperature in the dark. Aliquots of 15 μl were withdrawn after 3, 8, 16, 32, 60 and 120 min oxidation, and the oxidation was stopped by addition of 4 μl of 1 M sodium arsenite and frozen at −20°C. Formaldehyde produced was measured with a CTA reagent (chromotropic acid, E. Merck Ag., Darmstadt, Germany) by the method of SUZUKI and STROMINGER (1960). A specimen of *N*-acetylglucosamine was similarly reduced and oxidized as a reference. Maximum production of formaldehyde was attained by 60 min oxidation of test specimens in the present study.

10. *Determination of the average number of repeating units of teichoic acid*

The assay was based on the method of HAY *et al.* (1965). An aliquot of test specimen containing, 140 to 190 mμmoles of total phosphorus, was dried and redissolved in 30 μl of 0.2 M acetate buffer, pH 4.0. The solution was heated in boiling water in a sealed glass tube, and analyzed at 12 to 24 hr intervals for inorganic and total phosphorus. The amount of inorganic phosphorus released was plotted against the period of hydrolysis. The amount of unusually acid-labile phosphodiesters linking teichoic acid to the muramic acid residue in a glycan portion of the peptidoglycan was calculated on the basis of the amount of inorganic phosphorus released at the initial, more rapid, rate by extrapolation of the linear portion of the plots. The number of repeating units of teichoic acid was expressed as the reciprocal of the molar ratio of acid-labile phosphorus to total phosphorus.

11. *Anti-S. aureus rabbit serum*

Cells of *S. aureus* strain Copenhagen, grown as described above, were killed by heating at 60°C for 60 min and lyophilized. Two ml of the cell suspension (2.5 mg dry weight/ml) in solution containing 20,000 units of penicillin G and 100 mg of streptomycin hydrochloride were mixed with 4 ml of Drakeol (Pennsylvania Oil Co., Penn. USA) and 2 ml of Arlacel A (Atlas Powder Co., Ind., USA). Injections into the foot pads of adult rabbits, weighing about 3 kg, of 0.25 ml each of the above immunizing antigen, were made three times at weekly intervals. The rabbits received intravenous booster injections of 2 ml of a physiological saline suspension (2 mg/ml) of heat-killed cells on the third and fourth weeks. One week after the last injection, the animals were bled. The immune serum specimens obtained were mixed with sodium azide of a final concentration of 0.1% and stored at 4°C.

RESULTS

1. *Inhibition of phage 3C by cell walls of S. aureus strain Copenhagen*

The inhibitory activity of a pronase-treated cell wall specimen against phage 3C was studied in the broth or Ca-broth assay system, using a phage concentration of 1.3×10^3 PFU/plate and cell wall concentrations ranging from 0.04 to 5 μg (0.024 to 3 mμmoles organic phosphorus)/plate. Fig. 1 shows dose-response relations, obtained by plotting the per cent reduction in PFU after 30 min incubation against the logarithm of the amount of cell walls. These relations show that the per cent reduction in PFU changed linearly with the logarithm of the amount of the cell walls used as an inactivating agent, in both the broth and Ca-broth assay systems. The values of ID_{50}, the dose capable of producing 50% reduction in PFU under the standard conditions, of pronase-treated walls were calculated from the relations in Fig. 1 to be 0.5 and 0.28 μg in the broth and Ca-broth systems respectively. The difference between the ID_{50} values obtained in the broth and Ca-broth systems was small, and the enhancing effect of Ca ions on the phage 3C inhibiting activity of receptor sites was not so marked in the cell walls, as in the case of soluble receptor substances described later.

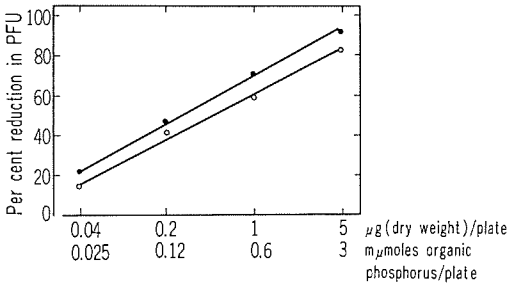

FIGURE 1 Inhibiting activity of cell walls of *S. aureus* strain Copenhagen upon phage 3C (dose-response relation).
-○—○-: in broth, -●—●-: in Ca-broth
Incubation time: 30 min

2. *Attempt to solubilize a phage 3C receptor from cell walls by digestion with the L-11 enzyme (unfractionated)*[3]

Trypsinized cell walls (600 mg) were incubated with 300 units of the L-11 enzyme (unfractionated) in 120 ml of 0.0125M phosphate buffer, pH 6.8, at 20°C. At frequent intervals the optical density of the reaction mixture was determined. When the decrease in optical density reached 50% of the initial value, NaCl was added to part of the mixture at a final concentration of 0.5M to stop the enzyme action (KATO *et al.*, 1962). The remainder of the mixture was further incubated until maximum optical density reduction (97%) was obtained. The 50% and complete lysates were centrifuged twice at 16,000 *g* for two hr to remove intact cell walls and fragments as completely as possible. The second super-

3 In this experiment, a crude phage 3C stock and trypsinized walls prepared from a different lot of *S. aureus* culture from that used for preparation of pronase-treated cell walls were used. Therefore, results on inhibition by trypsinized and pronase-treated cell walls on phage 3C cannot be compared directly, although the activity of trypsinized walls seemingly was stronger than that of pronase-treated walls.

natants were concentrated to an appropriate volume with a rotary evaporator under reduced pressure at 40°C. The concentrated solutions were thoroughly dialyzed in cellulose tubing (size 8/32, 50F, Visking Co., USA) against deionized water, and the non-dialyzable portions were lyophilized. The materials, amounting to 18 and 62% of the cell walls respectively, were recovered from the 50% and complete cell wall lysates.

The inhibiting activities of these two preparations against phage 3C were studied in the broth assay system. As shown in Fig. 2, while the material obtained from the complete lysate did not show any detectable inhibitory activity upon phage 3C at a concentration of as much as 250 μg (containing 148 mμmoles organic phosphorus)/plate even after 180 min incubation, 10 μg (18 mμmole equivalents of organic phosphorus) of the non-dialysable 50 % lysate inhibited 50, 84 and 94 % of phage 3C during incubation periods of 30, 60 and 180 min, respectively.

It was thus found that the L-11 enzyme

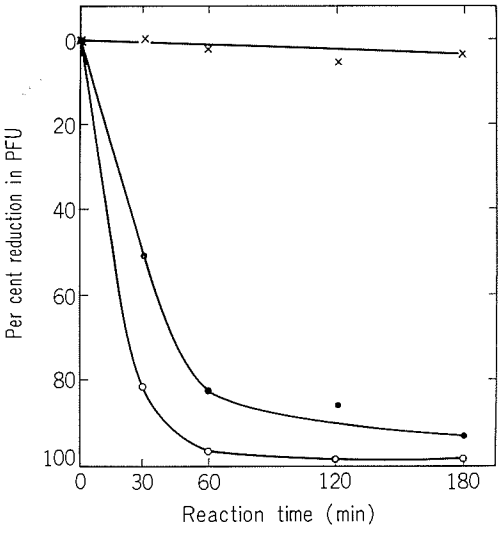

FIGURE 2 Phage 3C inhibiting activity of the lysates of cell walls solubilized by the L-11 enzyme (unfractionated).
-●—●-: 50 per cent lysate, -×—×-: 100 per cent lysate, -○—○-: cell walls

(unfractionated) could solubilize a phage 3C receptor site from the cell walls. However, the results obtained were not always reproducible, since if the lysis was stopped too early, the yield of active material was poor, and if the lysis proceeded too far, the inhibiting activity of the lysate was almost completely lost.

3. *Isolation of phage 3C receptor sites from the cell walls using L-11 endopeptidase*

The fact that the cleavage of pentaglycine cross bridge precedes the splitting of *N*-acetylmuramyl-L-alanine linkages during lysis of *S. aureus* cell walls by the L-11 enzyme (unfractionated) (KATO and STROMINGER, 1968), together with the findings described in the preceding section, suggest that an enzyme preparation exhibiting only the bridge splitting enzyme activity would be better for solubilizing phage 3C receptor sites in the cell walls than an enzyme specimen showing the amidase as well as the bridge splitting enzyme activities. An attempt was therefore made to solubilize phage 3C receptor sites by hydrolysis of the cell walls with the L-11 endopeptidase which was devoid of the *N*-acetylmuramyl-L-alanine amidase (see materials and methods section).

1) Solubilization of the cell walls by the L-11 endopeptidase

A 1,000 mg specimen of the pronase-treated cell walls was solubilized with 200 units of the L-11 endopeptidase preparation in 400 ml of 0.0125M phosphate buffer, pH 8.0, at 37°C. Aliquots of 500 μl, were withdrawn from the digestion mixture at appropriate intervals, and heated at 100°C for 2 min to inactivate the residual enzyme. Fig. 3 illustrates the results of estimations of optical densities and free amino groups in these specimens. In parallel with the decrease in optical density, free amino groups increased from 315 mμmoles/mg (0.63 mole/mole of total glutamic acid residues in the walls) at 0 time to 780 mμmoles (1.56 moles) at the time of maximum lysis after 48 hr incubation. No detectable liberation of free reducing groups occurred during the

lysis. Analyses of N-terminal and C-terminal amino acids (Fig. 4) revealed that the L-11 enzyme liberates 0.87 mole of N-terminal glycine, 0.36 mole of C-terminal alanine and 0.51 mole of C-terminal glycine per mole of total glutamic acid by its bridge splitting enzyme activities. No significant release of N-terminal alanine occurred, indicating that there was no cleavage of *N*-acetylmuramyl-L-alanine linkages during solubilization of the walls.

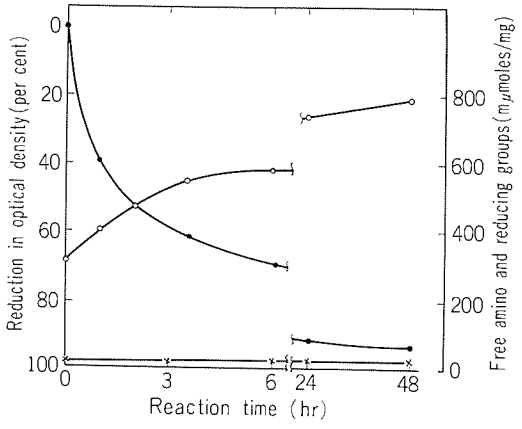

FIGURE 3 Lysis of *S. aureus* cell walls with liberation of free amino groups by digestion with the L-11 endopeptidase.
-●—●-: optical density, -○—○-: free amino groups, -×—×-: reducing groups

2) Isolation of a phage 3C receptor substance by fractionation of the L-11 endopeptidase digest of the cell walls

The cell wall digest which had been incubated for 48 hr in the preceding experiment was centrifuged at 16,000 *g* for one hr to sediment insoluble materials (117 mg, 11.7% of the starting cell walls). The sediment was washed once with 10 ml of deionized water by centrifugation. The supernatant and washing water were combined and centrifuged again at 16,000 *g* for one hr. The supernatant was concentrated to 30 ml with a rotary evaporator in a water bath at 40°C and centrifuged once more.

The solubilized cell wall materials thus obtained were shown by a preliminary experiment to have a definite inhibitory activity toward phage 3C. They were submitted to gel filtration on a Sephadex G-50 column

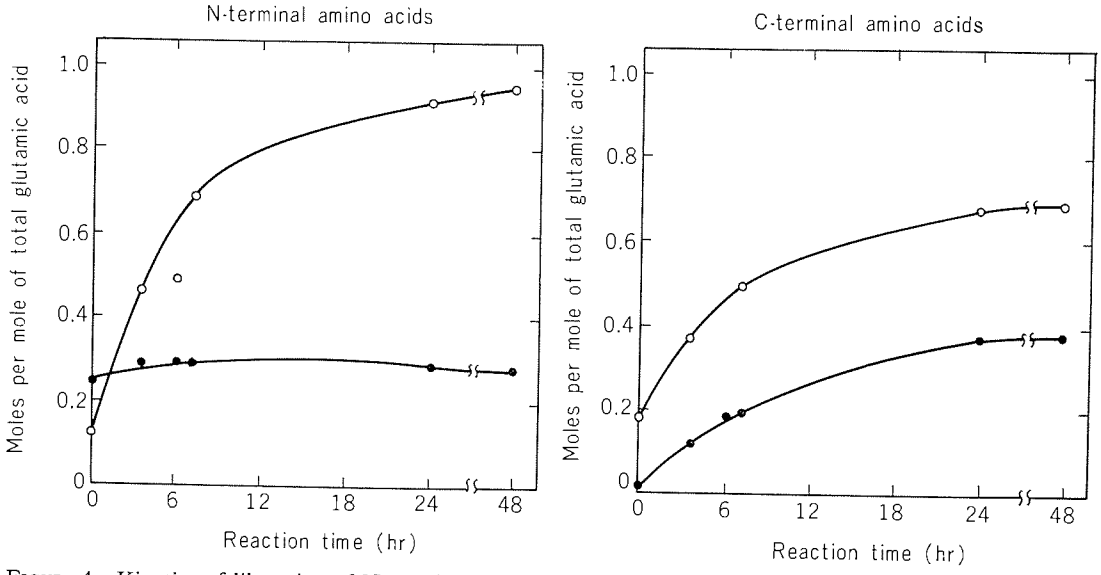

FIGURE 4 Kinetics of liberation of N-terminal and C-terminal amino acids during solubilization of cell walls with the L-11 endopeptidase.
-○—○-: glycine, -●—●-: alanine

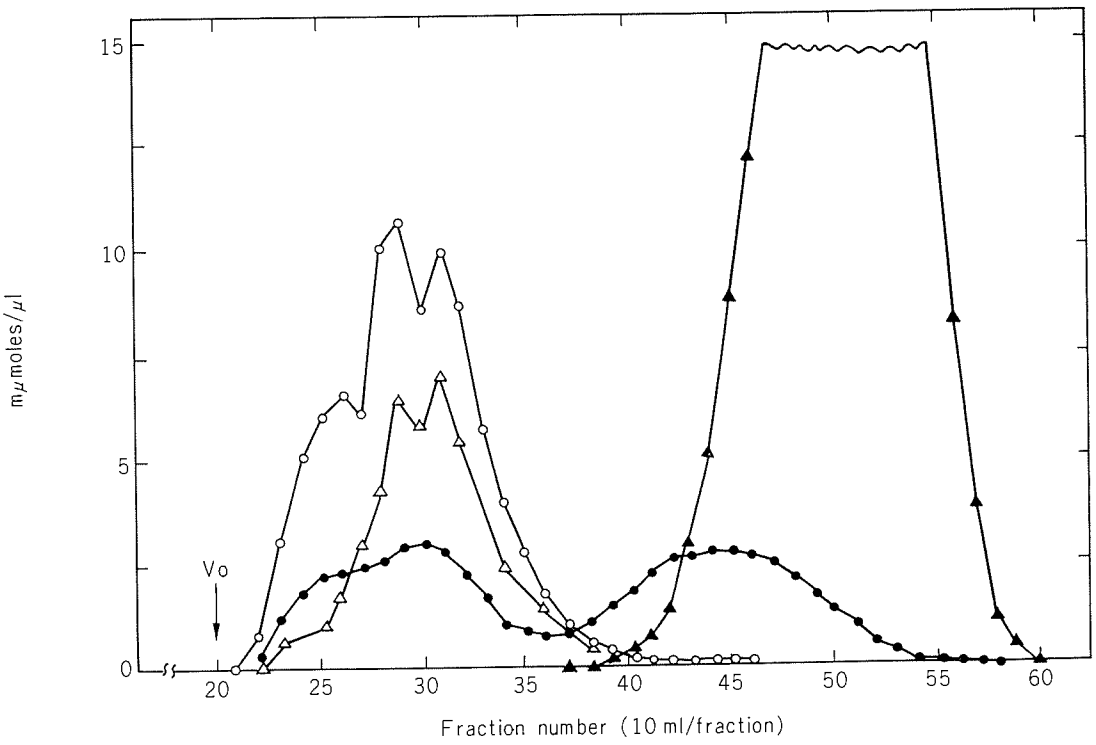

FIGURE 5 Fractionation on columns of Sephadex G-50 and G-25 in series of cell walls solubilized by the L-11 endopeptidase.
-○—○-: hexosamines, -△—△-: organic phosphorus, -▲—▲-: inorganic phosphorus, -●—●-: free amino groups

(1.83 $cm^2 \times 98$ cm) connected in series with a Sephadex G-25 column (3.48 $cm^2 \times 97$ cm). Elution was performed with deionised water, and fractions of 10 ml each were collected at a flow rate of 50 ml/hr. As shown in Fig. 5, a higher molecular weight fraction, which contained almost all of the hexosamines and organic phosphorus in the cell wall lysate applied on the columns, was clearly separated from a lower molecular weight fraction containing materials with amino groups. Aliquots of fractions 26, 29 and 31, which contained 60, 300 and 300 mμmoles of organic phosphorus, respectively, were tested for their inhibitory activities toward phage 3C, and shown to produce 23, 67 and 62% reduction in PFU respectively, in the broth assay system with an incubation period of 30 min. No activity was found in fraction 45.

Fractions 22 to 36 were combined, concentrated to about 5 ml, and applied on an ECTEOLA-cellulose column (1.33 $cm^2 \times$ 33 cm). The column was developed first with deionized water and then with a linear gradient of increasing molarity of LiCl, pH 5.0, as illustrated in Fig. 6. Fractions were collected at a rate of 11 ml/tube/15 to 30 min, and aliquots were analyzed for hexosamines, organic phosphorus and free amino groups. No materials were eluted with deionized water. Fraction differing in their relative contents of the three constituents analyzed, were partially separated from each other, by linear gradient elution with LiCl. Fractions 22, 24, 26 and 28 were thoroughly dialyzed against deionized water to remove

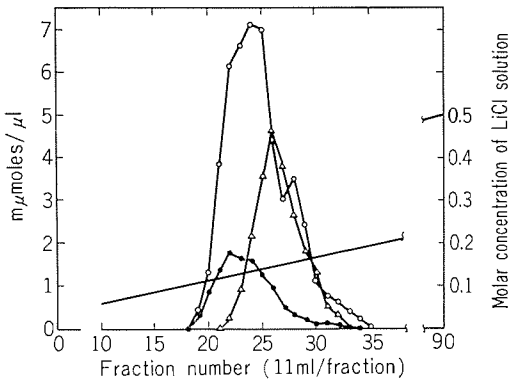

FIGURE 6 Chromatography of the higher molecular weight fraction of the cell walls solubilized by the L-11 endopeptidase with an ECTEOLA-cellulose column.

-○—○-: hexosamines, -△—△-: organic phosphorus, -●—●-: free amino groups

LiCl and their phage 3C inhibiting activities were tested in the broth and Ca-broth assay systems. While fraction 22, containing practically no organic phosphorus, was totally inactive, the other test fractions showed a definite inhibitory activity. The phage 3C inhibiting activities of the fractions seemed to be correlated with the content of organic phosphorus, but not of hexosamine (Table 1). This table also shows that addition of $CaCl_2$ to the assay system markedly increased the phage inhibiting activity of the test fractions.

To purify a receptor substance from the active fractions, fractions 23 to 36 were combined and dialyzed against deionized water. The contents of the dialysis tube were concentrated to about 6 ml, and applied to a column of ECTEOLA-cellulose similar to that used previously. The column was developed first with 520 ml of deionized water and then by concave gradient elution with 370 ml of 0.05 M LiCl, pH 5.0 in the mixing chamber and 185 ml of 0.2 M LiCl, pH 5.0 in the reservoir. However, since no materials were eluted in this way, the column was then eluted with 0.25 M LiCl, pH 5.0, at a flow rate of 5 ml/tube/10 min. Practically all of organic phosphorus and the bulk of the hexosamines and free amino groups applied on the column were eluted as a narrow band with a maximum around fraction 64 (Fig. 7). Fractions 62 to 70 were combined, thoroughly dialyzed against deionized water and concentrated to 8 ml.

TABLE 1 *Phage 3C inhibiting activity of fractions separated by gel filtration and ECTEOLA-cellulose column chromatography from the walls solubilized by the L-11 endopeptidase*

Fraction No.	Dose (mμmole equiv.)/plate		Per cent reduction in PFU					
			In broth			In Ca-broth		
	Organic phosphorus	Hexosamine	60[a]	120	180	60	120	180
22	16	534	0	0	0	—[b]	—	—
	32	1,068	—	—	—	2	4	0
24	134	440	0	0	2	58	70	69
	464	1,525	37	42	78	87	92	91
26	232	220	—	—	—	81	86	88
	464	440	37	47	68	98	98	98
	928	880	—	—	—	99	99	99
28	232	305	—	—	—	98	99	100
	464	610	43	53	81	—	—	—

a Incubation period in min

b Not tested

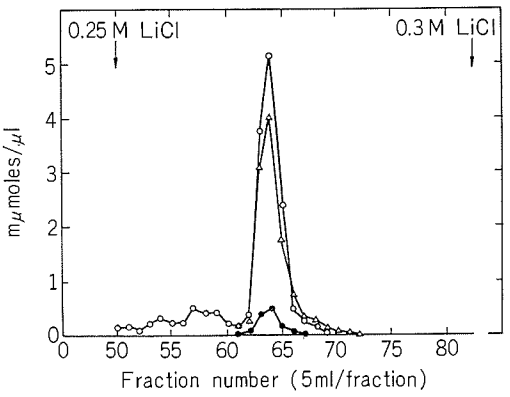

FIGURE 7 Rechromatography on ECTEOLA-cellulose column of phage 3C inhibiting fractions separated by ECTEOLA-cellulose column chromatography from cell walls solubilized with the L-11 endopeptidase.

-○—○-: hexosamines, -△—△-: organic phosphorus, -●—●-: free amino groups

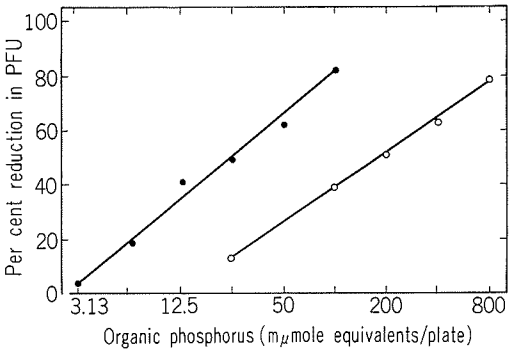

FIGURE 8 Inhibiting activity of the BS-B fraction isolated from the cell walls solubilized by the L-11 endopeptidase upon phage 3C (dose-response relation).

-○—○-: in broth, -●—●-: in Ca-broth
Incubation time: 30 min

This concentrated solution is referred to as the BS-B fraction. As an inactive control, fraction 22 in Fig. 6, was dialyzed and is termed fraction BS-A. Seventy, 50 and 31% of the organic phosphorus, hexosamines and free amino groups respectively, were recovered from the cell walls in the BS-B fraction.

3) Inhibitory activity of the BS-B fraction upon phage 3C

The inhibitory activities of various amounts of BS-B fraction against a constant number of phage 3C were tested in the broth and Ca-broth assay systems. Fig. 8 shows that the extent of inhibition, expressed as the per cent reduction in PFU, is directly proportional to the logarithm of the amount of the BS-B fraction added, in both assay systems. The ID_{50} of the BS-B fraction against phage was calculated from the curves presented in Fig. 8 to be approximately equivalent to 200 and 25 mμmoles of organic phosphorus in the broth and Ca-broth systems, respectively. The phage 3C inhibiting ability of the BS-B fraction was enhanced nearly 8 times by the presence of $CaCl_2$ in the assay systems. In addition, a small, but significant difference was noticed between the slopes of the dose-response relations in broth and Ca-broth. The Ca-broth assay system gave a somewhat steeper line than the broth assay system.

Thus with L-11 endopeptidase, it was possible to isolate phage 3C receptor sites in a soluble and purified form. It should be pointed out, however, that the potency of the solubilized receptor was estimated to be only one hundredth of that of the cell walls, on the basis of the organic phosphorus content, even in the presence of Ca ions. Prolongation of the incubation period from 30 min to 3 hr only slightly increased the per cent reduction in PFU by the BS-B fraction: increases of from 62 to 82, from 39 to 53, and from 13 to 30% reduction were recognized with BS-B fraction equivalent to 400, 100 and 25 mμmoles of organic phosphorus, respectively, in the broth system.

4) Chemical entity of the BS-B fraction

The results of chemical analyses of the BS-B and BS-A (as a reference) fractions are sumarized in Table 2. Both fractions contained muramic acid, alanine, glutamic acid, lysine and glycine in the approximate molar ratio of 1:2:1:1:2.5. The amino-terminal amino acid was glycine, and alanine and glycine residues were C-terminal, in both fractions. However, there was a marked difference, between the BS-B

TABLE 2 *Chemical properties of the active BS-B and inactive BS-A fractions*

Fraction	Chemical composition							N-terminal		C-terminal	
	Muramic acid	Glucosamine	Alanine	Glutamic acid	Lysine	Glycine	Organic phosphorus	Alanine	Glycine	Alanine	Glycine
BS-B	1.0	6.4	1.9	1.0	1.2	2.7	6.2	tr[a]	0.80	0.56	0.30
BS-A	1.1	1.1	1.9	1.0	1.1	2.3	tr	tr	0.92	0.73	0.45

Data are expressed as molar ratios to glutamic acid.
a Trace amounts

and BS-A fractions in their contents of organic phosphorus and glucosamine, indicating that the BS-B fraction contained teichoic acid, whereas fraction BS-A was almost free from teichoic acid.

The number of repeating units in teichoic acid in the BS-B fraction was determined by the method of HAY *et al.* (1965) to be about 31, on the basis of the results in Fig. 9. A purified teichoic acid preparation described later was analyzed as a reference. It was found that this preparation consisted, on the average, of 9 repeating units.

Terminal amino acid analyses indicated that most of the pentaglycine cross bridges were opened in both the BS-B and BS-A fractions, suggesting that these glycopeptides were interconnected through glycosidic linkages in a glycan moiety. Therefore, the average chain length of a glycan moiety in the BS-B and BS-A fractions was estimated by the method of TIPPER, STROMINGER and ENSIGN (1967). Table 3 shows that the glycan portions of both the BS-B and BS-A fractions consisted of about five disaccharide units. With the BS-B fraction, a correction was made for the formaldehyde produced from the terminal ribitol residues in teichoic acid by periodate oxidation. Since the chain length of teichoic acid in the BS-B fraction was about 31 repeating

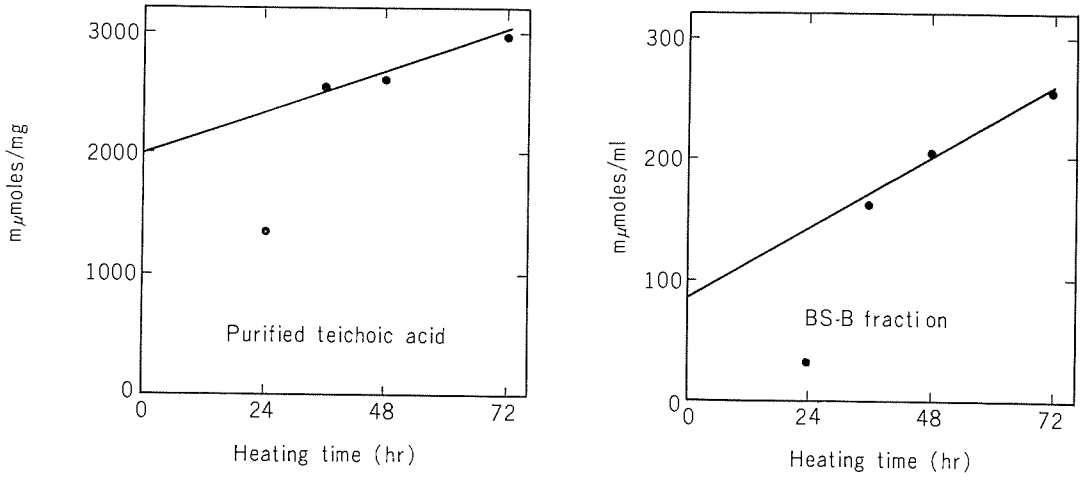

FIGURE 9 Liberation of inorganic phosphorus from the acid-labile phosphodiester linkages of the BS-B fraction and the purified teichoic acid by heating at 100°C in 0.2 M acetate buffer, pH 4.0.
Organic phosphorus/inorganic phosphorus (molar ratio)
Purified teichoic acid: 1,788/200=8.9 the BS-B fraction: 18,650/800=30.8

TABLE 3 *Estimation of average chain lengths of a glycan portion of the fractions isolated from the cell walls solubilized by the L-11 endopeptidase or Chalaropsis (B) enzyme*

Test material	Formaldehyde (mμmoles) liberated (A)[a]	Formaldehyde (mμmoles) to be liberated from teichoic acid portion (B)[b]	Formaldehyde (mμmoles) to be liberated from glycan portion (A-B)	Muramic acid (mμmoles) (C)	Ratio (C/A−B)[c]
BS-A	424	0	424	2,100	4.95
BS-B	1,280	602	678	3,000	4.42
Chala-H_2O	600	0	600	100	1.00
Chala-A	320	2	318	704	2.21
Chala-B	695	100	595	621	1.04

a By periodate oxidation of the specimens reduced with borohydride.
b This was calculated on the assumption that one mole of ribitol (terminal) per 31 moles of organic phosphorus liberates formaldehyde by periodate oxidation (see text).
c This ratio represents the average number of repeating disaccharide units.

units, as described above, 0.2 mole of formaldehyde was assumed to be produced per 6.2 moles of organic phosphorus. This value was substracted from the value obtained by periodate oxidation of the BS-B fraction.

These results indicate that the BS-B fraction, a phage 3C receptor substance solubilized from the cell walls by the L-11 endopeptidase, is a complex of highly polymerized teichoic acid and glycopeptide mainly interconnected by glycosidic linkages. The glycopeptide portion of the BS-A fraction was essentially the same as that in the BS-B fraction, except that it was devoid of teichoic acid, and it was totally inactive against phage 3C. This indicates that teichoic acid is an essential component of the 3C phage receptor site.

5) Serological reactivity of the BS-B fraction

This was examined by the ring precipitin test with anti-*S. aureus* cell rabbit serum. The BS-B fraction gave a definite positive reaction at a concentration of as low as 0.5 mμmole of organic phosphorus (0.61 mμmole hexosamine) equivalent/ml. However, the BS-A fraction only gave a positive reaction when a solution containing as much as 100 mμmole equivalents of hexosamine was used as the antigen.

4. *Isolation of phage 3C receptor sites from the cell walls using Chalaropsis (B) enzyme*

1) Solubilization of the cell walls by the Chalaropsis (B) enzyme

A sample of 100 mg of pronase-treated cell wall specimen was solubilized with the aid of 80 units of the Chalaropsis (B) enzyme by incubation in 20 ml of 0.025 M acetate buffer, pH 4.7, at 37°C for 10 hr. Determinations were made on the optical density, free reducing groups and amino groups in 320 μl aliquots of the reaction mixture which were withdrawn at appropriate intervals and heated at 100°C for 2 min. Lysis of the walls was accompanied by release of reducing groups without any detectable liberation of free amino groups (Fig. 10). The increase in reducing groups (as *N*-acetylglucosamine) amounted to 385 mμmoles/mg cell walls (0.8 mole/mole of total glutamic acid) after maximum lysis.

Solubilized cell walls were centrifuged three times at 16,000 *g* for one hr to remove insoluble material as completely as possible (dry weight of the total insoluble material, 6.1 mg). The supernatant fluid was thoroughly dialyzed against deionized water. The non-dialyzable

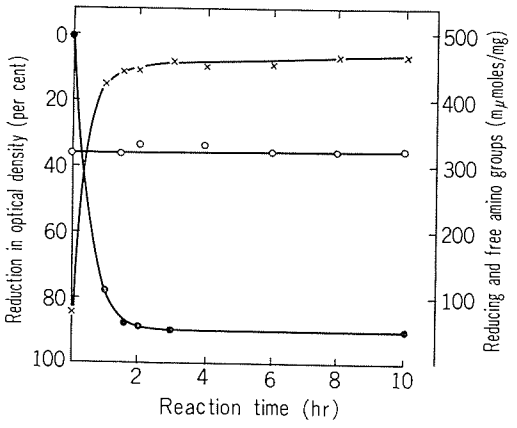

FIGURE 10 Lysis of *S. aureus* cell walls with liberation of reducing groups by digestion with the Chalaropsis (B) enzyme.
-●—●-: optical density, -×—×-: reducing groups, -○—○-: free amino groups

portion was recentrifuged and lyophilized (77.8 mg). A preliminary assay showed that this solubilized material had a definite inhibitory activity upon phage 3C.

2) Fractionation of solubilized materials

Solubilized materials were submitted to chromatography on an ECTEOLA-cellulose column in the same way as described in section 3–2) (Fig. 11). A peak fraction (Chala-H_2O) containing hexosamines but not organic phosphorus, was eluted with deionized water. On linear gradient elution with increasing concentrations of LiCl, two additional peak fractions were separated. Fractions 21 to 24, and 27 to 50 were separately pooled and termed the Chala-A and Chala-B fractions, respectively. Each was dialyzed against deionized water to remove LiCl. The Chala-H_2O and -A fractions were concentrated and lyophilized, yielding 20.7 mg (41% of the non-dialyzable portion) and 4.2 mg (8.3%) of material, respectively. The yield of the Chala-B fraction, which was not lyophilized, was calculated to

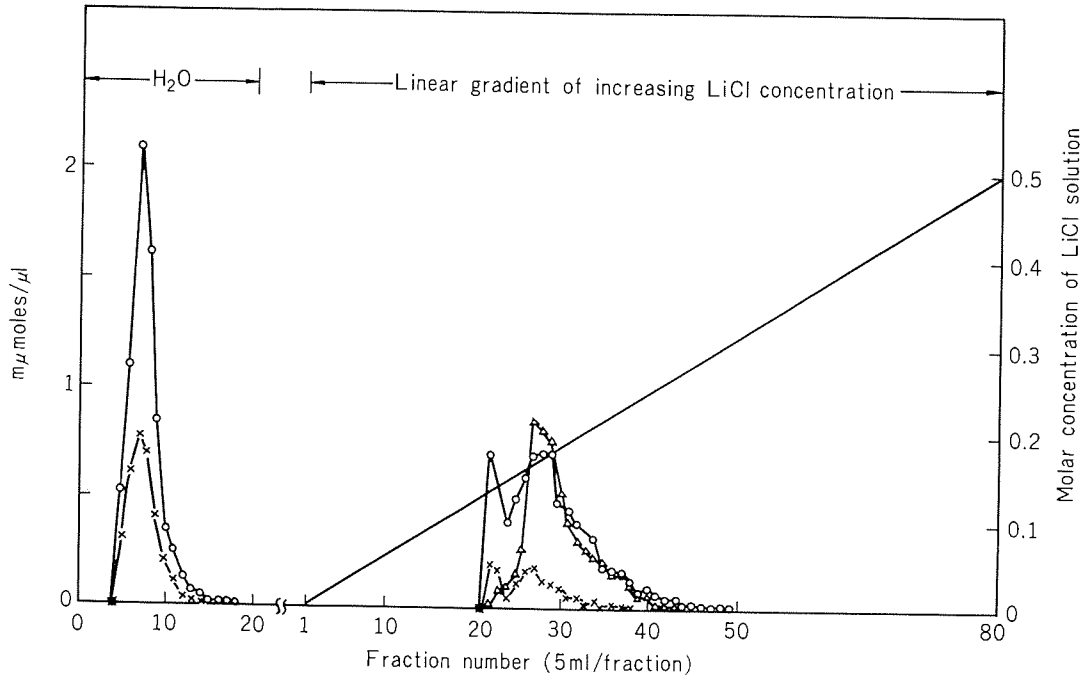

FIGURE 11 Chromatography of the non-dialysable portion of the cell walls solubilized by the Chalaropsis (B) enzyme on an ECTEOLA-cellulose column.
-○—○-: hexosamines, -△—△-: organic phosphorus, -×—×-: reducing groups

TABLE 4 *Phage 3C inhibiting activity of fractions seprated by ECTEOLA-cellulose column chromatography from the walls solubilized by the Chalaropsis (B) enzyme*

Fraction	Does (mμmole equiv.) / plate		Per cent reduction in PFU after 30 min incubation	
	Organic phosphorus	Hexosamine	In broth	In Ca-broth
Chala-H_2O	0	570	0	5
Chala-A	0	414	8	8
Chala-B	200	210	55	90

be 36% on the basis of the hexosamine content.

3) Inhibitory activities of the fractions isolated from the Chalaropsis enzyme digest

Table 4 summarizes the results of assays of the phage 3C inhibitory activities of the three fractions described above. Only the Chala-B fraction had definite inhibitory activity against phage 3C. The ID_{50} values of the Chala-B fraction were calculated to be 150 and 24 mμmole equivalents of organic phosphorus in the broth and Ca-broth assay systems, respectively, from the dose-response relations in Fig. 12. Thus the Chala-B fraction had the same order of phage 3C inhibiting activity as the BS-B fraction, and its inhibitory activity was markedly enhanced by the presence of Ca ions.

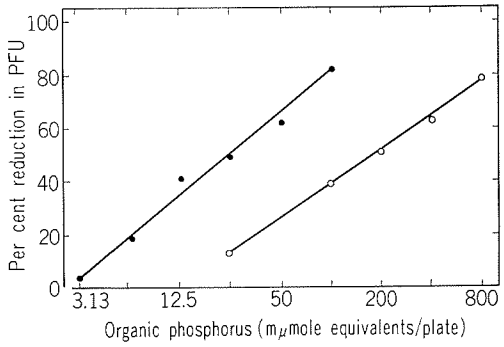

FIGURE 12 Inhibiting activity of the Chala-B fraction isolated from the cell walls solubilized by the Chalaropsis (B) enzyme upon phage 3C (dose-response relations).
-○—○-: in broth, -●—●-: in Ca-broth
Incubation time: 30 min

4) Chemical entity of the Chala-B fraction

Analyses showed that the Chala-H_2O, -A and -B fractions all contained one mole of glutamic acid, lysine and muramic acid, 2 moles of alanine and 5 moles of glycine, as shown in Table 5. Differences between the fractions were noticed in their contents of glucosamine and organic phosphorus: while the Chala-B fraction contained as much as 4 to 5 moles each of organic phosphorus and glucosamine per mole of glutamic acid, the other two fractions had little or no organic phosphorus and only about one mole of glucosamine per mole of glutamic acid (Table 5). The average chain lengths of the glycan portion[4] were estimated to be 1.0, 2.2 and 1.0 in the Chala-B, -A and -H_2O fractions, respectively (Table 3). Terminal amino acid analyses of the Chala-B fraction showed that only small fractions of the alanine and glycine residues were located at N- or C-terminals, indicating the presence of extensive numbers of pentaglycine cross bridges in this fraction. The length of the repeating units of teichoic acid in the Chala-B fraction could not be determined, since the amount of the fraction available was very limited. It can be concluded from the analytical data mentioned above that the Chala-B fraction is a complex of teichoic

4 A correction for formaldehydogenic groups of teichoic acid was made on the assumption that the number of repeating units of teichoic acid in the Chala-B fraction was the same as that in the BS-B fraction.

TABLE 5 *Chemical properties of the active Chala-B and inactive Chala-H_2O and -A fractions*

Fraction	Chemical composition							N-terminal		C-terminal	
	Muramic acid	Glucosamine	Alanine	Glutamic acid	Lysine	Glycine	Organic phosphorus	Alanine	Glycine	Alanine	Glycine
Chala-B	0.9	4.8	2.2	1.0	1.1	4.7	4.5	0.14	0.11	tr[b]	0.12
Chala-H_2O	0.8	1.0	1.9	1.0	1.1	4.9	0	—[a]	—	—	—
Chala-A	1.1	1.3	1.9	1.0	1.2	5.0	0.1	—	—	—	—

Data are expressed as molar ratios to glutamic acid.
a Not tested *b* Trace amounts

acid and polymerized disaccharide-peptide interconnected by pentaglycine cross bridges.

5) Serological reactivity of the Chala-B fraction

In the ring precipitin test the Chala-B fraction gave a positive reaction up to a concentration of 0.82 mμmole equivalent of organic phosphorus/ml with anti-*S. aureus* cell rabbit serum.

5. *Changes in the phage 3C inhibiting activity of the BS-B and Chala-B fractions by enzymatic hydrolysis*

To elucidate the minimum structure responsible for the phage 3C inhibition, the soluble receptor substances obtained in the foregoing studies were subjected to enzymatic treatment, and consequent changes in the inhibiting activities were investigated.

1) BS-B fraction

A specimen of the BS-B fraction, containing 7,460 mμmoles of organic phosphorus, was hydrolyzed by incubation with 24 units of the L-11 enzyme (unfractionated) in 400 μl of 0.01 M phosphate buffer, pH 8.0. Another specimen of the same size was incubated in 400 μl of 0.025 M acetate buffer, pH 4.7, containing 20 units of Chalaropsis (B) enzyme. The reaction was continued at 37°C for 48 hr in both cases. Changes in terminal groups and phage 3C inhibitory activity during the enzymatic hydrolysis were studied. The results are summarrized in Tables 6 and 7.

Treatment of the BS-B fraction with the L-11 enzyme (unfractionated) was accompanied by liberation of 0.8 mole of N-terminal alanine per mole of glutamic acid with a small release of N-terminal glycine, and resulted in a marked decrease in the phage 3C inhibiting activity. Hydrolysis with the Chalaropsis (B) enzyme, which liberated about 1.2 mole of reducing groups (as glucosamine) per mole of glutamic acid, did not cause any significant decrease in the phage inhibiting activity.

2) Chala-B fraction

Specimens of Chala-B fraction, each containing 4,000 mμmoles of organic phosphorus, were treated with 10 units of the L-11 enzyme (unfractionated) or 4 units of the L-11 endopeptidase, in 500 μl of 0.0125 M phosphate buffer, pH 8.0, at 37°C for 24 hr.

It can be seen from Tables 6 and 7 that half the muramyl-L-alanine linkages of the Chala-B fraction, were split off by hydrolysis with the L-11 enzyme (unfractionated) and it lost its phage 3C inhibiting activity completely. However, only about half the pentaglycine cross bridges of the Chala-B fraction were opened by treatment with the L-11 endopeptidase, and it retained most of its phage inhibiting activity.

Thus it seems that the glycan portion with teichoic acid and the peptide moieties must be linked together by *N*-acetylmuramyl-L-alanine bonds for exhibition of phage 3C inhibiting activity, but polymerization of glycopeptides

TABLE 6 *Analyses of terminal groups liberated during hydrolysis of the BS-B and Chala-B fractions by the L-11 enzyme (unfractionated), L-11 endopeptidase or Chalaropsis (B) enzyme*

Fraction	Treatment with	Reducing groups (as glucosamine)	Increase (moles/mole glutamic acid) in			
			N-terminal		C-terminal	
			Alanine	Glycine	Alanine	Glycine
BS-B	L-11 enzyme (unfractionated)	0.05	0.79	0.18	—[a]	—
	Chalaropsis enzyme	1.20	—	—	—	—
Chala-B	L-11 enzyme (unfractionated)	—	0.54	0.91	0.50	0.34
	L-11 endopeptidase	—	tr[b]	0.54	0.31	0.28

a Not determined *b* Trace amounts

TABLE 7 *Changes in phage 3C inhibiting activity of the BS-B and Chala-B fractions by enzymatic treatment*

Fraction	Treatment with	Does (mμmole equiv. organic phosphorus)/plate	Per cent reduction in PFU after 30 min incubation	
			In broth	In Ca-broth
BS-B	None	373	55	84
		187	52	84
		93		63
		47		45
	L-11 enzyme (unfractionated)	373	0	35
		187		[a]
		93		20
		47		14
	Chalaropsis enzyme	373	34	74
		187		72
		93		58
		47		39
Chala-B	None	800	90	
		400	56	
		200		86
	L-11 enzyme (unfractionated)	800	0	
		400		0
	L-11 endopeptidase	800	58	
		400	46	
		200		69

a Lost by accident.

TABLE 8 *Irreversibility of phage 3C inhibition by the BS-B fraction and cell walls*

Test material	Per cent reduction in PFU after 30 min incubation in Ca-broth		
	Control without L-11 enzyme treatment (tube 3)	Treatment by L-11 enzyme	
		Before phage adsorption (tube 1)	After phage adsorption (tube 2)
BS-B (226 mμmole equiv. organic phosphorus)	78	19	71
Cell walls (24 μg)	98	9	98

through either glycosidic linkages or pentaglycine cross bridges is not necessary for manifestation of the activity as phage 3C receptors.

6. *Irreversibility of phage 3C inhibition by the BS-B fraction*

This was demonstrated using the L-11 enzyme (unfractionated) in the following way.

Specimens, each containing 280 mμmoles of organic phosphorus, were incubated with (tube 1) or without (tube 2 and 3) 0.33 unit of the L-11 enzyme (unfractionated) in 80 μl of 0.01 M Tris-HCl buffer, pH 7.2. After one hr at 37°C, tubes were heated at 100°C for two min, and mixed with phage 3C (10^3 PFU) and $CaCl_2$ to give a final concentration of 0.01 M. The mixtures (volume 335 μl) were further incubated at 37°C for 30 min to allow adsorption of phage 3C by BS-B fraction. The mixture in tube 2 was then incubated with 0.33 unit of the L-11 enzyme (unfractionated) at 37°C for 30 min to test whether adsorbed phage 3C was liberated by destruction of the structure essential to the phage inhibition (the total volume at this stage was 615 μl and the concentration of $CaCl_2$ was 0.005 M). Tubes 1 and 3 were similarly incubated, but without the L-11 enzyme. Then 5 μl of $CaCl_2$[5] solution (final Ca concentration 0.02 M) was added to inhibit the enzyme action, and the number of PFU in 500 μl aliquots of each of the tubes was assayed. A similar experiment was performed with cell walls: 30 μg specimens of the walls were treated with 0.2 unit of the L-11 enzyme (unfractionated) before or after incubation with phage 3C. A control experiment showed that the L-11 enzyme preparation *per se* did not affect phage 3C.

As shown in Table 8, phage 3C which had been bound by the BS-B fraction or cell walls could not be recovered in an active form when the linkages between the glycan and peptide moieties in the phage-bound BS-B fraction or cell walls were opened (this was shown by the release of N-terminal alanine during enzymatic hydrolysis) and the structure essential to the phage 3C inhibiting activity was degraded. The irreversibility of inhibition of phage 3C by the BS-B fraction or cell walls was thus established.

The limited amount of the Chala-B fraction available made it imposible to study the irreversibility of phage 3C inhibition by this fraction.

7. *Absence of phage 3C inhibiting activity in a purified teichoic acid fraction*

A crude teichoic acid or a peptidoglycan fraction was prepared from the pronase-treated cell walls as described in a previous paper (SUGINAKA *et al.*, 1967) except that each of trichloroacetic acid extracts obtained was immediately extracted with ethylether to remove trichloroacetic acid.

Since chemical analyses demonstrated that this crude preparation was " contaminated " with a considerable amount of peptidoglycan,

5 In this experiment the assay was made in 0.01 M Tris-HCl buffer (pH 7.2) containing 0.01 M $CaCl_2$, since the activity of the L-11 enzyme was markedly inhibited in 0.02 M $CaCl_2$-broth.

a specimen of 100 mg was digested by 200 units of the L-11 enzyme (unfractionated) in 18 ml of 0.0125 M phosphate buffer, pH 8.0. After incubation for 18 hr at 37°C, during which time 486 mμmoles of free amino groups/mg were liberated, the incubation mixture was concentrated to about 5 ml, and applied on columns of Sephadex G-50 and G-25 connected in series. Fig. 13 shows that fractions 23 to 36 contained practically all the organic phosphorus, but were scarcely contaminated by substances with amino groups. These fractions were pooled and concentrated to about 8 ml. A pure teichoic acid fraction (52.5 mg) was precipitated from this concentrated solution by addition of 40 ml of ethanol in the

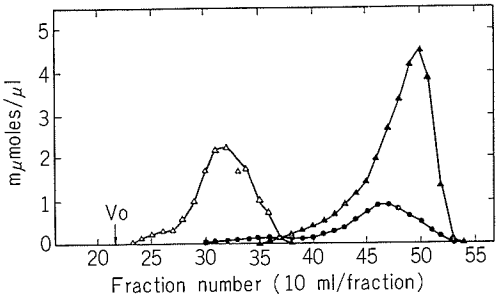

FIGURE 13 Separation of purified teichoic acid from the crude teichoic acid fraction treated with the L-11 enzyme (unfractionated) by filtration through columns of Sephadex G-50 and G-25 connected in series. -△—△-: organic phosphorus, -▲—▲-: inorganic phosphorus, -●—●-: free amino groups

presence of 1% (v/v) acetic acid and 10% (w/v) sodium acetate. This purified material was found to have the following composition (mμmoles/mg): organic phosphorus 1,788, hexosamine 1,475, total amino groups 750 and free amino groups 81 (the corresponding figures in the crude preparation were 1,760, 1,863, 3,350 and 444, respectively).

The teichoic acid and peptidoglycan fractions were tested for thier inhibitory activities against phage 3C. While 114 and 455 μg specimens of the crude fraction produced 24 and 70% reduction in PFU respectively on 30 min incubation in broth, neither purified teichoic acid (400 μg) nor peptidoglycan (320 μg containing 42 mμmoles organic phosphorus) showed any inhibitory effect on 3 hr or 30 min incubation in Ca-broth.

8. *Action of the receptor substances solubilized by cell wall lytic enzymes on phages other than 3C*

The possible inhibitory activities of the BS-B fraction and a non-dialyzable portion of the Chalaropsis (B) enzyme digest were studied against the following phages: lytic phage 3B of strain Copenhagen, and phage 42D and 53 which produce lytic infection in strain Duncan and strain 3528 respectively, but not in strain Copenhagen.

The cell walls were capable of inhibiting all the phages tested, irrespective of the ability of the phages to produce lytic infection in strain

TABLE 9 *Inhibiting activities against various staphylococcal bacteriophages of the cell walls or soluble phage 3C receptor substances of S. aureus strain Copenhagen*

Test material	Does (mμmole equiv.)/plate		Per cent reduction in PFU after 30 min incubation in Ca-broth			
	Organic phosphorus	Hexosamine	3C	3B	42D	53
Cell walls	15		97	98	99	89
	0.12		40	12	61	50
BS-B	800		92	0	0	0
BS-A		534	0	0	0	0
Non-dialyzable portion of walls solubilized by Chalaropsis enzyme	400		66	0	0	0
	200		54	0	0	0
Purified teichoic acid	800		0	0	0	0

TABLE 10 *Inhibitory activity of whole cells of three staphylococcal strains, in which the configuration of the N-acetylglucosamine residues in the teichoic acid moiety differ, toward phage 3C, 42D and 53*

Phage	Per cent reduction in PFU after 30 min incubation in Ca-broth								
	strain Copenhagen (3C/3B/55+)			strain Duncan (42D)			strain 3528 (53/83A/77)		
	50[a]	5	0.5	50	5	0.5	50	5	0.5
3C	80	17	6	99	91	8	48	6	0
42D	98	62	10	99	93	53	99	91	25
53	74	58	0	91	65	10	78	76	6

a mg whole cell/plate

Copenhagen. However, neither of the phage 3C receptor substances solubilized by the L-11 endopeptidase or Chalaropsis (B) enzyme had any detectable inhibitory activity toward phage 3B or phages 42D and 53, as indicated in Table 9.

9. *Test for Involvement of N-acetylglucosamine residues in the teichoic acid moiety in phage inhibition*

In a recent stydy, COYETTE and GHUYSEN (1968) demonstrated that the inhibiting activity against phage 3C, 71 and 77 decreased considerably on partial elimination of the *N*-acetylglucosamine residues from the teichoic acid moiety of the walls of *S. aureus* strain Copenhagen. Accordingly, the participation of the *N*-acetylglucosamine residues in the teichoic acid portion in the phage inhibitory activity was studied. Thus the inhibition of phages 3C, 42D and 53 by the whole cells of three strains, Copenhagen, Duncan and 3528, in which the configurations of the *N*-acetylglucosaminyl substitution of the D-ribitol units of the teichoic acid moiety differ were examined. NATHENSON *et al.* (1966) reported that the cell wall teichoic acid of strain Copenhagen was mainly a polymer of 4-*O*-β-*N*-acetyl-D-glucosaminyl-D-ribitol-5-phosphate and some 15% of the *N*-acetylglucosaminly substituents have the α-configuration.[6] Moreover, he showed that strains Duncan and 3528 contained practically all their substituent *N*-acetylglucosamine residues in β- and α-linkages, respectively.

Table 10 shows no significant correlation between the inhibitions of phages 3C, 42D and 53 by the whole cells of the three strains and the configuration of the *N*-acetylglucosamine residues in their teichoic acid moieties or the susceptibility of these strains to lytic infection by the test phages.

These results appear to be inconsistent with the observation of COYETTE and GHUYSEN (1968). Therfore, the effect of elimination of of *N*-acetylglucosamine residues in the teichoic acid moiety of the cell walls or the BS-B fraction of strain Copenhagen by *exo*-β-*N*-acetylglucosaminidase from pig epididymis was reinvestigated with the present assay system.

One hundred μg (containing 61.4 mμmoles of organic phosphorus) of the walls and 746 mμmole equivalents of the BS-B fraction (dried) were dissolved in 200 and 400 μl, respectivly, of stock solution of *exo*-β-*N*-acetylglucosaminidase in 0.01 M citric acid—0.02 M NaOH buffered saline containing 0.002% bovine serum albumin (2× Crystallized, Nutri-

6 TORII, KABAT and BEZER demonstrated that the α- and β-substituents were on separate teichoic acid molecules in strain Copenhagen.

TABLE 11 *Effect of treatment of cell walls or BS-B fraction with exo-β-N-acetylglucosaminidase upon their phage 3C inhibiting activity*

Test material	Treatment	Per cent reduction in PFU after 30 min. incubation in Ca-broth
Cell walls	None	92
(4.2 μg/plate)	*exo*-β-*N*-acetylglucosaminidase	93
BS-B	None	82
(311 mμmole equiv. organic phosphorus / plate)	*exo*-β-*N*-actylglucosaminidase	88

tional Biochemicals Corp., USA). On incubation at 37°C for 48 hr, 0.36 and 0.32 mole of the *N*-acetylglucosamine residues per mole of organic phosphorus were liberated from the walls and BS-B fraction, respectively. The phage 3C inhibiting activities of the walls and BS-B fraction after *exo*-β-*N*-acetylglucosaminidase treatment, were tested in the Ca-broth assay system (Table 11). Partial elimination of the *N*-acetylglucosamine residues of the teichoic acid moiety from the walls or the BS-B fraction did not decrease the inhibition of phage 3C. Thus the results of COYETTE and GHUYSEN could not be confirmed under the present experimental conditions, although the extent of elimination of *N*-acetylglucosamine residues was definitely lower in the present study than in that of COYETTE and GHUYSEN.

DISCUSSION

Many attempts have been made to solubilize the phage receptor sites from the cell walls of Gram-positive bacteria including *S. aureus*. However, results were generally unsuccessful. SALTON (1956 and 1957) reported that if the walls of *Bacillus megaterium* and *Micrococcus lysodeikticus* were solubilized with egg-white lysozyme prior to the addition of bacteriophage, no inactivation of the phage occurred. The loss of the phage adsorbing activity of *M. lysodeikticus* walls by lysozyme treatment was also reported by BRUMFITT (1960). Although VIDAVER and BROCK (1966) succeeded in isolation of active receptor materials against phage P3 from spheroplasts of *Streptococcus faecium* produced with lysozyme, all attempts to isolate receptor material without first making spheroplasts yielded either material which could not be solubilized, or material which was extractable but highly unstable.

Recently, COYETTE and GHUYSEN tried to solubilize the phage inhibiting substance from the walls of *S. aureus* strain Copenhagen with *Myxobacter* AL enzyme which hydrolyzes both peptide linkages in pentaglycine bridges and amidic *N*-acetylmuramyl-L-alanine linkages (1968). A preparation of glycan-teichoic acid complex isolated from a lysate of cell walls digested with the AL enzyme exhibited definite fixation activities toward phages 3C, 71 and 77, but the fixation was reversible.

The only successful attempt to solubilize the phage receptor sites of the cell walls with lytic enzymes seems to be that of KRAUSE (1957) on group C hemolytic streptococci. He demonstrated that a group C carbohydrate prepared from a *Streptomyces albus* digest of group C streptococcal cell walls inactivated group C phages, but not group A phages. Extensive attempts with crude enzymatic extracts of group A cell walls and group A carbohydrate to inactivate group A phages were not successful in demonstrating phage inactivation.

The present results clearly indicate that all the three main components of *S. aureus* wall peptidoglycan, that is, glycan, peptide subunits and teichoic acid, must be linked together to inhibit phage 3C irreversibly. Neither teichoic acid nor glycopeptides obtained by trichloroacetic acid extraction or enzymatic hydrolysis inactivated phage 3C. These findings are in good agreement with the studies of MORSE (1962) and MATTHEW and ROSENBLUM (1967), but seem to be contradictory to the conclusion of CAMERON and his colleagues (ROSATO and CAMERON, 1964; CAMERON and OSBORNE, 1967). They reported that a receptor substances

against phage 77 isolated by sonication of the walls of strain 77 was a mucopeptide containing no organic phosphorus, and demonstrated that a cell wall precursor accumulating in strain 77 on treatment with vancomycin was capable of both attachment to, and inactivation of phage 77. It should be pointed out here that MATTHEW and ROSENBLUM (1967) found that the residue remaining insoluble after extraction of purified cell walls of strain Copenhagen with trichloroacetic acid, and consisting mainly of mucopeptide, was capable of moderate phage adsorption, but the adsorption was reversible.

The necessity of *N*-acetylmuramyl-L-alanine linkages at the junction between the glycan and the peptide for manifestation of the inhibiting activity against phage 3C was clearly demonstrated by the fact that treatment of both active fractions, BS-B and Chala-B, with the L-11 enzyme with *N*-acetylmuramyl-L-alanine amidase activity was accompanied by marked loss of phage inhibiting activity. In this connection it is interesting that COYETTE and GHUYSEN (1968) suggested that to fix phages, 4-*O*-β-*N*-acetylglucosaminly-D-ribitol units of teichoic acid must possess a definite configuration which, in native cell walls, is imparted by the binding of teichoic acid to the supporting peptidoglycan. No information on the participation of *N*-acetylglucosamine residues themselves in the phage inhibiting activity, were obtained in the present study. The reason for the discrepancy between our results and those of COYETTE and GHUYSEN is uncertain at present.

Polymerization of the glycopeptide-teichoic acid complex by chains of alternating muramic acid and glucosamine in a glycan portion or by pentaglycine bridges between the carboxyl group of the D-alanine residue of one peptide subunit and the ε-amino group of the lysine residue of the neighboring subunit does not seem to be essential to the receptor site activity for phage 3C. This is implied by the observation that either treatment of the BS-B fraction with the Chalaropsis (B) enzyme or digestion of the Chala-B fraction with the L-11 endopeptidase did not affect the phage 3C inhibiting activity.

Though we solubilized the 3C phage receptor sites, the specific activity of the isolated receptor substances (expressed as mμmole equivalents of organic phosphorus required to produce 50 per cent reduction in PFU under the standard assay conditions) was much less than that of the cell walls in the starting material. This was so even in the presence of $CaCl_2$ which enhanced the activity of the soluble receptor substances. The low efficiency of either BS-B or the Chala-B fraction as phage 3C receptor sites may be an inevitable consequence of deviation from the natural conformation of native cell walls, caused by solubilization. If the phage inhibiting ability of soluble receptor substances could be enhanced, studies on them would be easier. One possibility is the adsorption of soluble receptor materials onto the stromata of red blood cells as reported in the study of TAYLOR and KWIATKOWSKI (1963) on the Vi-receptor—Vi-phage system. In the precent study, $CaCl_2$ was added to the assay system at an appropriate concentration, because of the enhancing effects of Ca ions upon the adsorption of T-series phages onto *Escherichia coli* (PUCK, GAREN and CLINE, 1952) and upon the inhibition of *S. aureus* phages by trypan blue (Jay, 1967). A marked enhancing effect of Ca ions on the phage 3C inhibiting activity of the soluble receptor substances, but not upon the activity of cell walls, was recognized. The mechanism of this enhancement is unknown.

Neither BS-B nor the Chala-B fraction caused any detectable inhibition of staphylococcal bacteriophages (3B, 42D and 53) other than 3C. In addition, none of their fractions isolated from the cell walls solubilized by the L-11 endopeptidase or Chalaropsis (B) enzyme inhibited these phages. This result was rather unexpected because the cell walls themselves caused unspecific inhibition of different phages, irrespective of the test phage to lyse the organism from which the wall preparation of the cell walls, as reported by

MORSE (1962) in a study on the interactions between the walls of *S. aureus* strain NYH-6 and a variety of different staphylococcal bacteriophages. A possible explanation for this would be that a more definite configuration is required in receptor sites for phages other than phage 3C and that this is held in native cell walls. The problem of solubilization and characterization of the receptor substances for other staphylococcal phages remains to be elucidated.

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