

STUDIES ON THE CHEMISTRY AND IMMUNOCHEMISTRY OF CELL WALLS OF STAPHYLOCOCCUS AUREUS*

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The studies described in this communication were designed to explore further the chemical and antigenic structure of cell walls of *Staphylococcus aureus*. These investigations were performed utilizing a virulent 80/81 strain of *S. aureus* as the prototype organism.

Three aspects of cell wall structure and function were examined in detail; (a) the chemical composition and lysozyme susceptibility of purified cell walls and mucopeptide; (b) the immunologic reactions of intact cell walls; and (c) the immunochemistry of a compound composed of ribitol phosphate and glucosamine which was isolated from the cell walls.

Materials and Methods

Organisms.—The strain of *Staphylococcus aureus* studied in most detail was originally isolated at New York Hospital from an infant with fatal staphylococcal pneumonia and was kindly supplied by Dr. Henry Scheinfeld. It was phage type 80/81 and was designated strain NYH-6. The other strains of staphylococci have been previously described (1). All were maintained on penassay agar slants. The liquid media utilized contained 1 per cent technical grade casamino acids (Difco), 0.5 per cent yeast extract (Difco), and 0.5 per cent glucose buffered to pH 7.0 with phosphate. The organisms were grown overnight at 37°C and then killed with phenol. The bacterial cells were harvested by centrifugation, washed with distilled water, and acetone-dried. Occasionally the pH of the cultures was controlled by the addition of sodium carbonate in the presence of phenol red. This procedure resulted in rapid attainment of maximum viable titer and the results presented were not affected by the use of bacteria cultured by this technique.

Preparation of Cell Walls.—The procedures utilized in the preparation of staphylococcal cell walls were as follows. 200 mg of dried bacteria, 5 ml of distilled water, 4 ml of No. 12 ballotini beads (Brinkmann Instruments Inc., Great Neck, New York), and one drop of tri-*n*-butyl phosphate were added to each cup of the Mickle disintegrator. The suspensions were subjected to maximal vibration for 20 minutes at 4°C. At the end of this time over 99 per cent of the cocci were disrupted as evidenced by the loss of both the Gram-stain reaction and phase denseness.

The beads were removed by filtration through coarse sintered glass and the suspensions were centrifuged at 12,800 *G* for 20 minutes at 4°C. The crude cell wall pellet was washed twice with distilled water and resuspended in phosphate buffer, pH 7.8, at a concentration

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equivalent to 100 mg/ml of starting material. Crystalline trypsin (Nutritional Biochemicals Corp.), 200 μ g/ml, and ribonuclease (Worthington), 100 μ g/ml, were added and the flasks incubated in the presence of toluene overnight at 37°C. The cell walls were then washed twice in distilled water and resuspended to volume in 0.01 N HCl. Pepsin (Nutritional Biochemicals Corp.), 100 μ g/ml, was added and the flasks were incubated at 37°C for 3 hours. This time period, shorter than that employed by other investigators, was chosen to avoid extensive extraction of acid-soluble cell wall components. The purified cell walls were then exhaustively washed with distilled water and dried from the frozen state. The yield of cell walls was 12 to 15 per cent of the starting material.

Analytical Procedures.—Phosphorus was determined by the method of Fiske and Subbarow (2), and nitrogen by the technique of Koch and McMeekin (3). Amino acids were determined by a modification of the method of Mandelstam and Rogers (4). Hexosamines were assayed by the Elson-Morgan procedure after hydrolysis of the samples in sealed ampules in 2 N HCl at 100°C for 3 to 4 hours (5). The amino sugars were separated on charcoal-celite columns (6). Lipid was determined by the method of Folch *et al.* (7).

Hydrolytic products were separated by chromatography on Whatman No. 1 paper. The solvent systems of most usefulness were: (a) *n*-butanol-acetic acid-water (4:1:5); (b) *n*-propanol-ethyl acetate-water (7:1:2); (c) isopropanol-formic acid-water (7:1:2); (d) *n*-butanol-pyridine-water (6:4:3). Compounds were detected with silver nitrate (8), ninhydrin, the hexosamine reagents, and the perchloric acid-ammonium molybdate reagent for phosphorus (9).

Paper and glass fiber electrophoreses were carried out in borate buffer at pH 10 (10). Ammoniacal silver nitrate was the developing reagent (11).

Cell Wall Agglutination Tests.—Sera were diluted twofold in a volume of 0.5 ml in plastic trays containing cups with a capacity of 2.5 ml (Linbro). Cell walls were brought into fine suspension by brief vibration in the Mickle disintegrator with No. 13 ballotini beads at a concentration of approximately 3 mg of cell walls/ml of physiologic saline. Two drops of the suspensions from a capillary pipette were added to each cup. The degree of agglutination was recorded after incubating the trays first at 37°C for 30 minutes, and then at room temperature for 1 hour. Refrigerating the trays overnight at 4°C did not lead to significant differences in the degree of agglutination.

Antisera were prepared by repeated intravenous injections of intact heat- or phenol-killed organisms, or cell walls into rabbits.

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RESULTS

Chemical Analysis of Cell Walls of NYH-6.—Only four amino acids (glycine, glutamic acid, alanine, and lysine), were demonstrated in the cell walls of NYH-6. Two amino sugars, glucosamine and muramic acid, and 2.1 per cent of organic phosphorus were also present (Table I). Lipid accounted for less than 0.5 per cent of the cell walls suggesting only minimal contamination of the preparations with bacterial membranes.

The nature of the organic phosphorus was then studied. Cell walls dissolved in 6 per cent perchloric acid did not show absorption peaks at 250 to 290 $m\mu$, indicating the absence of nucleic acid phosphorus. Ribitol phosphate and glycerophosphate have been demonstrated in the cell walls of many Gram-positive organisms (12), and acid hydrolysates of NYH-6 cell walls were examined for the presence of these compounds.

Cell walls were heated at 100°C in H₂SO₄ in sealed ampules under various conditions. The hydrolysates were brought to pH 5.2 with Ba(OH)₂ and after removal of BaSO₄ the samples were evaporated to dryness *in vacuo*.

When cell walls were heated in 0.1 N H₂SO₄ for 18 hours, two distinct components, in addition to the amino sugars, stained with silver nitrate in chromatographic solvent systems (a) and (b). One of these, of intermediate mobility, was identified as ribitol. The other, a rapidly migrating compound which stained with much less intensity, had the same R_f as anhydrosorbitol and glycerol.

In contrast, after heating in 2 N H₂SO₄ for 4 hours, the fast moving component stained more intensely than the ribitol moiety. This compound was shown to be anhydrosorbitol by electrophoretic analysis.

The phosphate compounds released during hydrolysis were examined in chromatographic solvent system (c). In addition to inorganic phosphorus, the

TABLE I
Chemical Analysis of Cell Walls of S. aureus NYH-6

Substance	Per cent of cell wall	μMoles/mg of cell wall
Alanine.....	14.0	1.57
Glycine.....	21.1	2.69
Lysine.....	11.7	0.81
Glutamic acid.....	23.6	1.60
Glucosamine (as <i>N</i> -acetylglucosamine).....	16.2	1.06
	(19.9)	—
Muramic acid (as <i>N</i> -acetylmuramic acid).....	9.1	0.36
	(11.5)	—
Phosphorus.....	2.1	0.67
Lipid.....	0.5	—

major component was ribitol phosphate. A small amount of anhydrosorbitol phosphate was also found. No glycerophosphate could be demonstrated.

It was therefore concluded that ribitol phosphate, and not glycerophosphate, was a component of NYH-6 cell walls.

Preparation and Properties of the Mucopeptide of Staphylococcus aureus-NYH-6.—It was of interest to dissect by chemical means, the ribitol phosphate from the amino sugar-amino acid portion of the cell walls. This was accomplished by a modification of the procedure described by Park (13).

NYH-6 cell walls at a concentration of 4 mg/ml in 5 per cent trichloroacetic acid (TCA) were heated at 90°C for 15 minutes. The residual material was isolated by centrifugation, washed with distilled water, and lyophilized.

32 per cent of the cell wall weight was solubilized which correlated with the observed reduction in optical density. Table II indicates the release of individual cell wall constituents during the extraction procedure. The most striking fea-

ture was the extensive release of phosphorus (94.1 per cent) and glucosamine (41.3 per cent). There was considerably less solubilization of the other components (9 to 16 per cent) and the molar ratios of these substances remained constant.

The residue was examined by both phase microscopy and electron microscopy. It was composed of material of the same size and shape as the cell walls but was slightly less dense. Structured residues composed solely of amino acid-amino sugar complexes have been isolated from cell walls of several bacterial species and have been termed the mucopeptide of the cell wall.

The Effects of Lysozyme upon the Cell Walls and the Mucopeptide of NYH-6.—The cell walls of NYH-6 were resistant to the lytic action of lysozyme, and

TABLE II
The Effect of Extraction with Hot TCA on the Cell Walls of NYH-6

Substance	Release by hot TCA	Molar ratio*	
		Cell wall	Residue after extraction (mucopeptide)
	<i>Per cent</i>		
Alanine.....	13.7	0.98	0.95
Glycine.....	10.1	1.68	1.68
Lysine.....	15.5	0.50	0.48
Glutamic acid.....	12.8	1.0	1.0
Glucosamine.....	41.3	0.66	0.37
Muramic acid.....	9.3	0.22	0.23
Phosphorus.....	94.1	0.42	0.03

* With respect to glutamic acid.

experiments were undertaken to ascertain if this was also true of the mucopeptide prepared by extraction with hot TCA.

Cell walls and mucopeptide were brought into suspension by brief vibration in the Mickle disintegrator. Suspensions at a concentration of 2 mg/ml in phosphate buffer (pH 7.0, $\mu = 0.1$), were incubated at 37°C in the presence of varying amounts of egg-white lysozyme. The optical densities of the mixtures at 650 $m\mu$ were recorded at intervals.

As noted in Fig. 1 after 60 minutes there was a reduction of only 11 per cent in the optical density of cell wall suspensions in the presence of 100 μg of lysozyme/mg of material. Prolonged incubation resulted in no further change.

In contrast, the mucopeptide was quite sensitive to the action of lysozyme. As little as 6 μg of lysozyme/mg of mucopeptide resulted in a 46 per cent reduction in optical density in 60 minutes. At higher lysozyme concentrations, the reaction rate was increased and became asymptotic after a 65 per cent decrease in opacity had occurred.

That the apparent changes were due to solubilization of material was confirmed by determination of the total hexosamine present in the supernatant fluids of the reaction mixtures. The release of total hexosamine was found to correlate with the diminution in optical density. Only 12 per cent of the hexosamine released was present as free acetylglucosamine indicating that complex units had been split off by the action of lysozyme. Similar results were obtained after the cell walls of two other strains of lysozyme-resistant *Staphylococcus aureus* were treated in the same fashion with hot TCA.

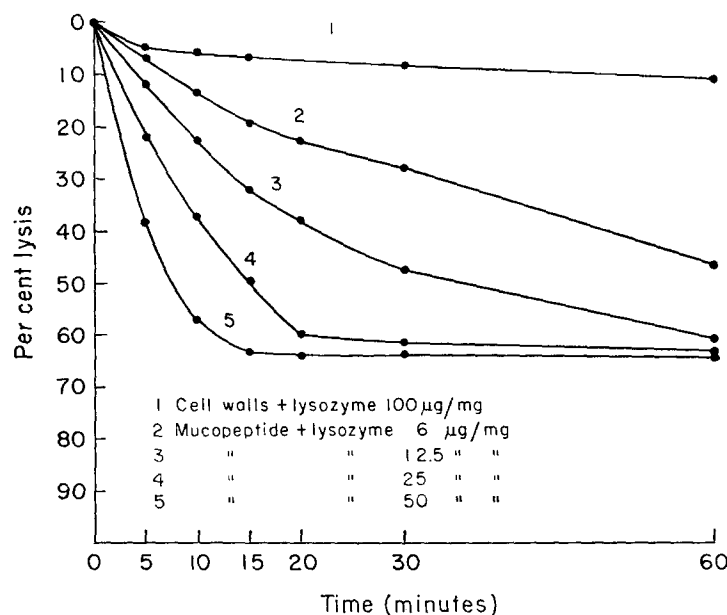


FIG. 1. The effect of lysozyme on the mucopeptide and cell walls of *S. aureus* strain NYH-6.

The effects on lysozyme sensitivity of extraction of cell walls of NYH-6 with hot and cold TCA were then compared.

Treatment of NYH-6 cell walls with 5 per cent TCA at 4°C for 48 hours resulted in the release of 63 per cent of the cell wall phosphorus. The cold TCA residue was resuspended after washing in distilled water at a concentration of 1 mg/ml of buffer. 50 µg of lysozyme/ml were added and the degree of lysis compared with that of intact cell walls and mucopeptide incubated with lysozyme under the same conditions.

After 60 minutes at 37°C, 10 per cent of the cell walls, 32 per cent of the cold TCA residue, and 65 per cent of the mucopeptide had been lysed. Thus the susceptibility of cell walls of NYH-6 to the action of lysozyme was correlated with the amount of organic phosphorus released.

The effect of hot TCA appeared to be relatively specific for staphylococci,

since extraction of cell walls of either *E. coli* K12 or Group C streptococci did not result in the formation of a lysozyme-sensitive residue.

The Occurrence of S. aureus Cell Wall Agglutinins.—A number of antisera prepared against various strains of *S. aureus* were tested for their ability to agglutinate purified cell walls of *S. aureus* strain NYH-6. Although numerous samples of normal rabbit serum did not agglutinate the cell walls, several sera prepared against both the homologous organism and *S. aureus* strains of differing phage types had agglutination titers ranging from 1:160 to 1:640. However, the production of cell wall agglutinins was not a regular occurrence. Thus, paired rabbits immunized with the same dose of either intact organisms or cell walls of strain NYH-6 showed marked variability in their ability to produce cell wall agglutinins.

The Serologic Relationship between S. aureus Cell Walls.—Because of the variability in agglutinin production, studies on cross-reactivity were performed by absorption of cell wall agglutinating serum with cell walls of different organisms. The absorbing dose of the heterologous cell walls was 5 to 10 times that amount of NYH-6 cell walls which would remove agglutinins under the experimental conditions employed.

Rabbit antiserum R-14 was prepared by injection of intact phenol-killed NYH-6 organisms. Suspensions of cell walls were added to the serum in conical centrifuge tubes. The final serum dilution was 1:10 and the concentration of absorbing material was 4 mg/ml. After incubation at 37°C for 30 minutes, the tubes were placed at 4°C for 18 hours. The cell walls were removed by centrifugation and the serum tested for the persistence of agglutinins.

As indicated in Table III, the serum agglutinin titer was reduced from 1:160 to less than 1:10 by absorption with cell walls of three other strains of *S. aureus* as well as by absorption with cell walls of the homologous organism. It was of interest that the mucopeptide of the cell walls of NYH-6 was incapable of removing cell wall agglutinins. Moreover, absorption with cell walls of *S. albus*, Groups A and C streptococci, and zymosan did not cause a significant reduction in titer.

Studies on the Reaction between S. aureus Antisera and S. albus Cell Walls.—Although agglutinins directed against *S. aureus* cell walls could not be removed by absorption of antisera with *S. albus* cell walls, several *S. aureus* antisera agglutinated cell walls of both species. The nature of this cross-reaction was explored in the following manner.

Antisera R-14 and Stovall-2, prepared against intact organisms of *S. aureus* strains NYH-6 and Stovall respectively, were absorbed with cell walls of strains of *S. aureus* and *S. albus*. The absorptions were carried out at a concentration of 4 mg of cell walls/ml of 1:10 serum dilution. The absorbed sera were then tested for their capacity to agglutinate both *S. aureus* and *S. albus* cell walls.

As noted in Table IV, absorption of *S. aureus* antisera with *S. aureus* cell walls, completely removed agglutinins against *S. aureus* cell walls, but had no

TABLE III

Effects of Absorption of S. aureus NYH-6 Antiserum by Cell Walls, Mucopeptide, and Zymosan on Agglutination of Cell Walls

Serum R-14 absorbed with	Agglutination of <i>S. aureus</i> NYH-6 cell walls						
	Reciprocal of final serum dilution						
	10	20	40	80	160	320	640
<i>S. aureus</i> * NYH-6 cell walls.....	0	0	0	0	0	0	0
" " NYH-3 " ".....	0	0	0	0	0	0	0
" " Stovall " ".....	0	0	0	0	0	0	0
" " 42E " ".....	0	0	0	0	0	0	0
<i>S. aureus</i> NYH-6 mucopeptide.....	++++	++++	+++	++	+	±	0
<i>S. albus</i> Greaves cell walls.....	++++	+++	+++	++	+	0	0
" " Prengel " ".....	++++	++++	+++	+++	++	±	0
" " McGafferey " ".....	++++	++++	+++	+++	++	±	0
Group A Streptococcal cell walls.....	++++	++++	+++	++	+	±	0
Group C " " " ".....	++++	++++	+++	++	+	±	0
Zymosan.....	++++	++++	+++	++	+	±	0
No absorption.....	++++	++++	+++	++	+	±	0

* Strains NYH-6 and NYH-3 are phage type 80/81. Strain Stovall is type 83 (VA₄) and 42E is the propagating strain for phage 42E.

TABLE IV

Effects of Absorption by Cell Walls on the Ability of S. aureus Antisera to Agglutinate S. aureus and S. albus Cell Walls

Serum	Prepared against	Absorbed with cell walls of	Reciprocal of agglutination titer against cell walls of			
			<i>S. aureus</i>		<i>S. albus</i>	
			Stovall	NYH-6	Greaves	Prengel
R-14	<i>S. aureus</i> NYH-6	<i>S. aureus</i> NYH-6	<10	<10	80	80
"	" " "	<i>S. albus</i> Greaves	160	160	<10	<10
"	" " "	No absorption	160	160	160	160
Stovall-2	<i>S. aureus</i> Stovall	<i>S. aureus</i> Stovall	<10	<10	160	80
"	" " "	<i>S. albus</i> Prengel	80	80	<10	<10
"	" " "	No absorption	160	160	320	80

significant effect on the ability of these sera to agglutinate *S. albus* cell walls. In like manner, absorption with *S. albus* cell walls removed only the agglutinins directed against cell walls of this species.

Preparation and Chemical Properties of the Ribitol Teichoic Acid of S. aureus NYH-6.—The serologic activity of the cell walls of strain NYH-6 prompted attempts to isolate soluble antigenic material from these walls.

Enzymatically treated cell walls of NYH-6 were extracted twice with 5 per cent TCA at 4°C for 48 hours. The residual cell walls were removed by centrifugation and the material which was precipitated from the supernatant fluid by 2 volumes of acetone was harvested by centrifugation, washed with acetone, and dried *in vacuo*. It was then purified by reprecipitation from aqueous solution with absolute ethanol.

The isolated substance was readily soluble in water and physiologic saline and solutions had no appreciable viscosity. Approximately 4 per cent was dialyzable.

TABLE V
Chemical Composition of the Teichoic Acid of S. aureus NYH-6

Component	Per cent
Na ribitol phosphate*	74.8
Acetylglucosamine	29.4
Total Amino Acids	1.3
Alanine	0.5
Glycine	0.5

* Calculated on the basis of 6.1 per cent organic phosphorus found.

The chemical composition of the compound is presented in Table V. The two major components were glucosamine and an organic phosphate compound which was shown to be ribitol phosphate. The phosphorus:glucosamine ratio was 1.5:1.

This cell wall substance was similar to those isolated from other strains of *S. aureus* and which have been termed ribitol teichoic acids. However, the teichoic acids isolated from other *aureus* staphylococci have contained significant amounts of alanine in addition to ribitol phosphate and glucosamine (14, 15). In contrast, the ribitol teichoic acid of *S. aureus* NYH-6 contained less than 2 per cent of total amino acids.

Serologic Activity of the Isolated Ribitol Teichoic Acid.—Although several *S. aureus* antisera precipitated the teichoic acid of NYH-6, the reactions for the most part were weak. It was therefore necessary to employ the more sensitive method of hemagglutination to demonstrate the presence of antibody. The teichoic acid was not fixed to sheep red cells, but was absorbed onto the surface of erythrocytes tanned by a procedure modified from that originally described by Boyden (16).

Sheep's blood in Alsever's solution was centrifuged and the erythrocytes were washed with saline buffered to pH 7.2 with phosphate. 0.1 ml of packed erythrocytes were then suspended in 4.0 ml of buffer, and 4.0 ml of a 1:20,000 solution of tannic acid in buffer was added slowly with stirring at room temperature. The suspension was incubated at 37°C for 10 minutes, and the cells were washed twice with buffer to remove excess tannic acid. The erythrocytes were suspended in 20.0 ml of buffer, and 2 mg of teichoic acid in 4.0 ml of saline was added. After incubation at 37°C for 90 minutes, the erythrocytes were centrifuged down, washed 3 times, and resuspended in 4.0 ml of buffer.

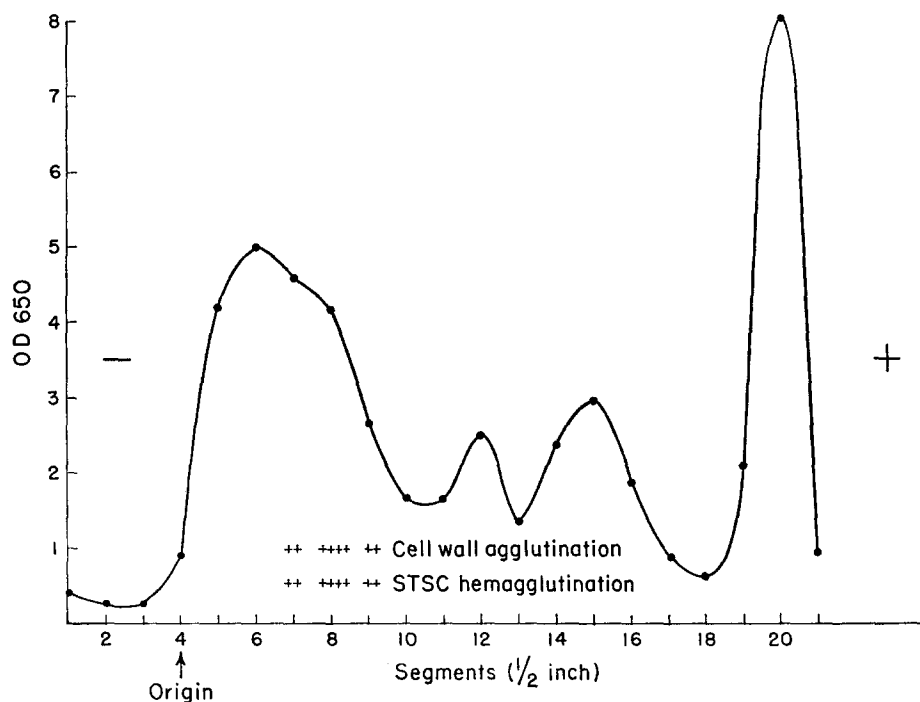


FIG. 2. The localization of STSC hemagglutinins and cell wall agglutinins in *S. aureus* antiserum.

All sera were inactivated by heating at 56°C for 30 minutes. The sera were diluted in the cups of plastic trays in a total volume of 0.5 ml. Two drops of the sensitized tanned sheep cells (STSC) were added to each cup. After thorough mixing, the trays were placed at 37°C for 30 minutes and then at room temperature for 1 hour. At the end of this time period the degree of hemagglutination was recorded.

Several of the *S. aureus* antisera had hemagglutinating titers of 1:320 to 1:2048, whereas none of 15 samples of normal rabbit sera were reactive at dilutions of 1:10.

The Nature of the Serum Component Reacting with Sensitized Tanned Sheep Cells (STSC).—Antiserum R-14 was fractionated by zone electrophoresis in

barbital buffer, (pH 8.6, $\mu = 0.1$), using polyvinyl chloride (pevikon) as the supporting medium. Half-inch segments were cut from the block, and the eluted protein measured and tested for both cell wall agglutinins and hemagglutinins.

As indicated in Fig. 2, both the NYH-6 cell wall agglutinins and the STSC hemagglutinins of serum R-14 were located in the electrophoretically fast moving portion of the gamma globulin. Immunoelectrophoresis of the isolated protein also indicated that the reactive material was fast moving gamma globulin.

The results with serum R-14 suggested that the hemagglutinins and cell wall agglutinins might be the same antibody. However, certain other *S. aureus* antisera reacted with STSC at high dilutions but did not react with cell walls of strain NYH-6 at dilutions as low as 1:10. This suggested that the immunologic activity of *S. aureus* cell walls was not entirely represented by the antigenic activity of the isolated teichoic acid. This supposition was supported by the ob-

TABLE VI
The Inhibition of STSC Hemagglutination by Cold TCA Extracts of Various Cell Walls

Extracts prepared from cell walls of	Highest dilution of extract inhibiting hemagglutination
<i>S. albus</i> Greaves.....	<1:2
" " Prengel.....	<1:2
" " Air.....	<1:2
<i>S. aureus</i> NYH-6.....	1:2048
" " Stovall.....	1:1024
" " O'Hara.....	1:1024

servation that it was difficult to effectively inhibit cell wall agglutination even with high concentrations of ribitol teichoic acid.

Inhibition of STSC Hemagglutination by Cold TCA Extracts of Cell Walls.— In order to study the distribution of the antigenic ribitol teichoic acid, cold TCA extracts of *S. aureus* and *S. albus* cell walls were tested for their ability to inhibit the hemagglutination reaction.

20 mg of cell walls were extracted in 2 ml of 5 per cent TCA for 36 hours at 4°C. the residue was removed by centrifugation and the supernatant fluids were extracted with ether in order to remove the TCA. 0.25 ml of the extracts was then serially diluted twofold, and 0.25 ml of a 1:20 dilution of *S. aureus* NYH-6 antiserum was added to each cup. After mixing, the trays were placed at 37°C for 30 minutes. Sensitized tanned erythrocytes were added and the hemagglutination reaction carried out as described above.

Extracts of *S. aureus* cell walls at dilutions between 1:1024 and 1:2048 completely inhibited hemagglutination. In contrast no inhibition occurred with the extracts of *S. albus* cells walls even at dilutions as low as 1:2 (Table VI). Under

the circumstances of this experiment 1 μg of teichoic acid completely inhibited hemagglutination.

The Antigenic Determinant of Ribitol Teichoic Acid.—The technique of hemagglutination inhibition was utilized to determine whether ribitol phosphate or *N*-acetylglucosamine was the antigenic determinant of teichoic acid isolated from *S. aureus* strain NYH-6.

0.25 ml of phosphate-saline buffer containing 2 μM of inhibitor was added to 0.25 ml of a 1:320 dilution of antiserum. The mixtures were incubated at 37°C for 45 minutes. Tanned erythrocytes sensitized with teichoic acid were then added and the degree of hemagglutination was ascertained.

TABLE VII
The Effect of Monosaccharide Inhibitors on STSC Hemagglutination

Inhibitors*	Degree of hemagglutination in 60 minutes
<i>N</i> -acetylgalactosamine.....	++++
<i>N</i> -acetylglucosamine.....	++
α -phenyl-acetylglucosamine.....	+++
β -phenyl-acetylglucosamine.....	+
Na ribitol phosphate.....	++++
	++++

* 2 μmoles of inhibitor added.

As indicated in Table VII, *N*-acetylglucosamine inhibited the hemagglutination reactions whereas the addition of equimolar concentrations of *N*-acetylgalactosamine or ribitol phosphate did not cause inhibition.

In an effort to determine the immunologically effective linkage of *N*-acetylglucosamine, the teichoic acid was incubated in the presence of β -*N*-acetylglucosaminidases from two different sources. Neither enzyme was capable of releasing free *N*-acetylglucosamine. However, two different preparations of α -phenyl-*N*-acetylglucosamine were not effective inhibitors of hemagglutination, whereas β -phenyl-*N*-acetylglucosamine was an effective inhibitor (Table VII).

The Quantitative Precipitin Reaction between Teichoic Acid and S. aureus Antiserum.—One of the antisera prepared against intact organisms of strain NYH-6 was found to produce a strong precipitin reaction with teichoic acid. The quantitative precipitin curve was then determined.

Various quantities of teichoic acid were added to 0.2 ml of antiserum in a total volume of 2.0 ml. The tubes were mixed and placed at 37°C for 30 minutes and then at 4°C for 18 hours.

The tubes were centrifuged, and the precipitates were washed 3 times with cold physiologic saline. The precipitates were dissolved in 0.1N NaOH, and aliquots were taken for determination of protein by the method of Lowry *et al.* (17).

Fig. 3 shows the precipitin curve obtained. In the region of antigen excess there was approximately 60 per cent inhibition of precipitation. The effects of the hexosamines and substituted hexosamines on the precipitin reaction were then studied.

5 μ M of inhibitor were added to 0.2 ml of rabbit antiserum in a volume of 1.5 ml. The tubes were incubated at 37°C for 45 minutes. 0.5 ml of saline containing a slight excess of teichoic acid was then added and the precipitin reaction was studied as described above.

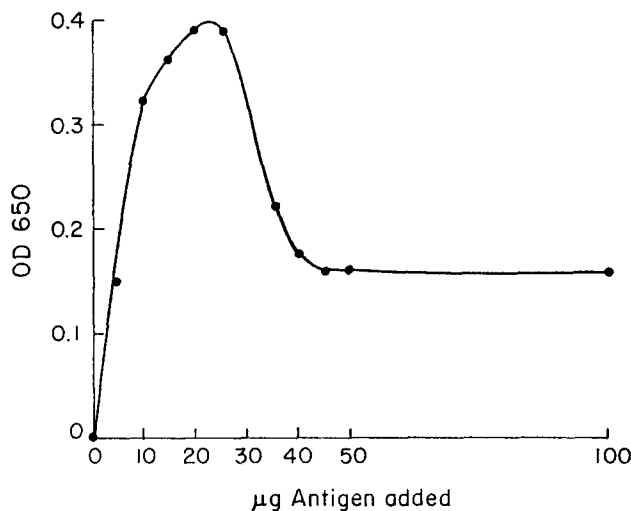


FIG. 3. Quantitative precipitin reaction of *S. aureus* antiserum and *S. aureus* teichoic acid.

Under the conditions employed, *N*-acetylgalactosamine did not inhibit the precipitin reaction. *N*-acetylglucosamine and α -phenyl-*N*-acetylglucosamine inhibited the reaction by 8 per cent and 6 per cent, respectively. In contrast the addition of β -phenyl-*N*-acetylglucosamine caused a 25 per cent reduction in the amount of specific precipitate. Thus, these observations supported the evidence obtained by hemagglutination-inhibition tests that β -linked *N*-acetylglucosamine was the antigenic determinant of the teichoic acid of strain NYH-6.

The Reaction between Human Serum and STSC.—Preliminary studies on the presence of hemagglutinating antibody directed against *S. aureus* teichoic acid in normal human sera have been performed. Of ten sera studied only four had hemagglutinating titers greater than 1:10. These reactions were generally weak, and did not exceed a titer of 1:80.

The serum of one patient with recurrent staphylococcal furunculosis was also studied. This patient had received autogenous vaccine and injections of staphylococcal toxoid as well as antibiotic therapy during the long course of his illness. The serum, while it did not agglutinate cell walls of NYH-6 at a dilution of 1:10, caused hemagglutination of STSC at a dilution of 1:1280. The serum protein components were separated by zone electrophoresis. As was the case with rabbit antiserum, the hemagglutinating activity was found only in the most negatively charged portion of the gamma globulin. Immuno-electrophoretic and sucrose density studies indicated that the human antibody was an electrophoretically fast moving 7S gamma globulin.

DISCUSSION

The cell walls of *Staphylococcus aureus* strain NYH-6 were shown to consist of four amino acids (glycine, lysine, alanine, and glutamic acid), two amino sugars (glucosamine and muramic acid), and ribitol phosphate. These findings were qualitatively the same as those noted by other workers utilizing different strains of *S. aureus*. However, the molar ratio glutamic acid: alanine in the NYH-6 strain was greater (18, 19).

The cell walls of strains of *Staphylococcus aureus* thus far studied have been shown to contain polymers of ribitol phosphate to which are appended side chains of glucosamine and alanine (20-22). This compound, ribitol teichoic acid, can be extracted from *S. aureus* cell walls by exhaustive treatment with trichloroacetic acid in the cold. A more efficient, but degradative method of extraction is brief treatment with hot TCA. This procedure removes approximately 95 per cent of the cell wall phosphorus. It has been suggested that the effect of acid treatment is to break a bond between the alanine portion of teichoic acid and the mucopeptide (19). Against this proposed mechanism for the linkage of the teichoic acids to the mucopeptide, at least in the NYH-6 strain, are the results obtained when cell walls of these organisms are extracted with hot 5 per cent TCA. Organic phosphorus and glucosamine are the only substances selectively removed (95 per cent and 41 per cent respectively). This indicates that alanine is most likely not responsible for the linkage of the teichoic acid to the mucopeptide.

The mucopeptide of cell walls of NYH-6 and of two other *S. aureus* strains, prepared by extraction with hot TCA, are susceptible to the action of lysozyme whereas the cell walls are resistant. Recently it has been reported that extraction of cell walls of *S. aureus* Copenhagen with 10 per cent TCA at 4°C for 3 weeks results in the formation of a lysozyme-sensitive residue (20). Shorter periods of extraction of NYH-6 cell walls in cold TCA results in partial lysozyme susceptibility which is correlated with partial release of cell wall phosphorus. It would thus appear that the removal of teichoic acid from the cell wall permits lysozyme to attack the glucosamine-muramic acid bond of the mucopep-

tide. This is another example in which a lysozyme-sensitive residue can be prepared from lysozyme-resistant cell walls (23, 24). It is possible that non-mucopeptide moieties confer lysozyme resistance by steric hinderance or absorption of the enzyme.

Although the existence of common staphylococcal antigens has long been suspected, their nature and localization have not been firmly established. In this investigation, utilization of fractionation techniques permitted antigenic dissection of staphylococci and it was possible to determine not only the existence of common antigens but also their localization. Thus, antisera prepared against intact cells of *S. aureus* agglutinate both *S. aureus* and *S. albus* cell walls. However, absorption of the antisera with the cell walls of one species removes only the agglutinins directed against cell walls of the homologous species. This indicates that the cross-reacting antigen present in *S. aureus* which stimulates the production of *S. albus* cell wall agglutinins is itself not a cell wall component of *S. aureus*.

One of the possible cross-reacting antigens is polyglycerophosphate. This substance is an "intracellular" component of *S. aureus*, and is also a component of the teichoic acid of the cell walls of *S. albus* (25, 26).¹

The teichoic acid of the cell walls of *S. aureus* strain NYH-6, consist almost entirely of ribitol phosphate and glucosamine. In contradistinction to the composition of teichoic acids isolated by others from different strains of *S. aureus*, alanine is not a major component (14, 15). The heterogeneity of teichoic acids with respect to alanine content also exists with respect to the nature of the linkage of *N*-acetylglucosamine. Variable amounts of α - and β -linked acetylglucosamine have been found not only in teichoic acids prepared from different strains of *S. aureus*, but also in teichoic acids prepared from different batches of the same organism (14).

The nature of the glucosamine linkage in the teichoic acid of strain NYH-6 is difficult to establish by enzymatic methods. Two preparations of β -glucosaminidase do not split off acetylglucosamine. Nevertheless immunochemical studies clearly demonstrate that β -linked acetylglucosamine is the antigenic reactant. This discrepancy may be due to the known inability of certain glucosaminidases to hydrolyze acetylglucosamine from compounds in which the proper linkage is known to be present (27).

Detection of antibodies directed against the isolated teichoic acid is facilitated by the use of sensitized tanned sheep cells (STSC). In parallel with the results on cell wall agglutination, cross-reacting antigens are located in the cell walls of *S. aureus*, but not in cell walls of *S. albus*.

Two observations make it apparent that the isolated teichoic acid is not the sole antigenic determinant of *S. aureus* cell walls: (a) the disparity in serum

¹ Recently, glycerophosphate and glucose have been identified as the components of the teichoic acid of the cell walls of a strain of *S. albus* (Prenzel) used in this investigation.

titers against STSC and cell walls; and (b) the difficulty in inhibiting cell wall agglutination with the isolated antigen. Since the method of extraction removes only 66 per cent of the ribitol teichoic acid, it is possible that the residual material differs in some manner from the extracted antigen. Perhaps this difference resides in the number of antigenically effective α -linked acetylglucosamine units.

The results presented in this study, in addition to those recently presented by others (15, 26) on the antigenic and immunologic role of *S. aureus* cell walls and teichoic acid, may permit accurate classification and more complete antigenic analyses of the staphylococcus species.

SUMMARY

The cell walls of an 80/81 strain of *Staphylococcus aureus* (NYH-6) contain alanine, glycine, glutamic acid, lysine, muramic acid, glucosamine, and ribitol phosphate.

94 per cent of the phosphorus and 41 per cent of the glucosamine are removed by extraction of the cell walls with hot 5 per cent TCA, but significant amounts of the other constituents are not extracted by this procedure.

The residue after hot TCA extraction (mucopeptide) is susceptible to lysozyme whereas the intact cell walls are resistant.

Staphylococcus aureus cell walls are agglutinated by *S. aureus* antisera. Agglutination of the cell walls of one *S. aureus* strain is inhibited by absorption of antisera with cell walls of other *S. aureus* strains but not by absorption with *S. albus* cell walls.

The ribitol teichoic acid can be isolated from cold TCA extracts of the cell walls. This compound consists almost entirely of ribitol phosphate and glucosamine.

The isolated teichoic acid of strain NYH-6 is readily fixed to tanned sheep erythrocytes and these sensitized cells are agglutinated by *S. aureus* antisera. Cold TCA extracts of cell walls of other strains of *S. aureus* inhibit hemagglutination whereas extracts of *S. albus* walls do not.

Studies on the inhibition of both hemagglutination and precipitation indicate that the antigenic determinant of *S. aureus* NYH-6 teichoic acid is β -*N*-acetylglucosamine.

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