CHEMICAL BASIS FOR AN IMMUNOLOGICAL SPECIFICITY OF A STRAIN OF STAPHYLOCOCCUS AUREUS*

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The structure and biosynthesis of the cell wall of *Staphylococcus aureus*, strain Copenhagen, have been investigated previously (*cf.* reference 1). Studies of the immunological specificity of this strain were undertaken for several reasons. The chemical nature of the surface structure (the cell wall) of this organism was under investigation and had been partially elucidated. If, as seemed likely, the cell wall contained immunological determinants, the chemical nature of structures responsible for this activity could be defined. Relatively little is known about the chemistry of the antigens in staphylococci or their relationship to such biological phenomena as immunity, allergy, phagocytosis, and intracellular lysis. Moreover, it seemed likely that immunological methods would prove useful in further studies of the biosynthesis of the cell wall of *S. aureus*. These have been the stimuli to the present work.

Rabbit antisera were prepared against whole organisms of *S. aureus*, strain Copenhagen, and were found to agglutinate cell walls prepared from this species. Hapten inhibition of this agglutination has led to the conclusion that the teichoic acid (*cf.* reference 2) in the cell wall (a polymer of ribitol phosphate which contains glycosidically linked *N*-acetylglucosamine and esterified D-alanine) contains a serologically active group. Among the earliest studies of the antigens in *S. aureus* strains were those of Julianelle and associates (3, 4). They isolated a substance termed polysaccharide A 25 years ago and showed that it was active both in precipitin tests with rabbit antisera and in inducing wheal and erythema in humans. A similar, but distinct substance, termed polysaccharide B, was isolated from *Staphylococcus albus* strains. In retrospect the described properties, as well as the method of isolation, of polysaccharides A and B indicate that these compounds were teichoic acids. In recent years, immunological activity of the polyglycerophosphate (which belongs to the family of teichoic acids, *i.e.* polyol phosphate polymers) found in several Gram-positive species has been demonstrated by McCarty (5). Since publication of our preliminary reports of the

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immunological activity of the ribitol teichoic acids in *S. aureus* (6, 7), several other investigators have reported serological activity of these compounds in *S. aureus* (8–10) and in other microorganisms (11). The relationship of the teichoic acids to polysaccharide A has been investigated in detail by Haukenes (9).

Methods and Materials

Immunization Procedure.—Suspensions of *S. aureus*, grown in nutrient broth, were killed by addition of 0.5 per cent formaldehyde and incubation for 48 hours at 37°C.[1] The dead cells were then removed by centrigugation and mixed with Freund's adjuvant. Using foot-pad injections, a total of 4 mg (dry weight) of formalized cells were injected into each rabbit. The rabbits were bled by cardiac puncture 1 month after immunization. They were then given booster injections with 1 mg of formalized cells intramuscularly, and bled a second time 5 days after the booster injection. The blood from the cardiac punctures was allowed to clot at room temperature for 1 hour and left at 3°C overnight. After centrifuging at low speed, the sera were poured off and recentrifuged at high speed. The lipid layer was removed by suctioning and the sera decanted.

Preparation of Purified Cell Walls.—5 ml of glass beads (Ballotini No. 12), 5 ml of a slurry of whole cells, and 4 ml 0.1 M phosphate buffer, pH 7.2, were added to the cups of a Mickle disintegrator and shaken for 2 hours. The beads were removed by filtration. The suspension was centrifuged and the pellet was washed several times. Trypsin (final concentration, 0.1 per cent) was added to this crude cell wall, the pH adjusted to 7.5, and the suspension incubated at 37°C for 12 hours to remove protein adhering to the cell walls. The suspension was again centrifuged and the cell walls washed three times with water. Purity of this cell wall preparation was examined after hydrolysis in 6 N hydrochloric acid in a sealed tube at 100°C for 12 hours. The hydrolysate was taken to dryness *in vacuo*, dissolved in a small volume of water, and spotted on Whatman No. 1 chromatography paper. Descending paper chromatography, using the organic phase of butanol:acetic acid:water (4:1:5), followed by staining with 0.3 per cent ninhydrin in water-saturated *n*-butanol, indicated the presence of only four amino acids, alanine, glutamic acid, lysine, and glycine, the four amino acids characteristic of the cell wall. The amount of any other amino acid was less than 1 per cent of the amount of alanine present, indicating the virtual absence of contaminating cytoplasmic protein.

To maintain a homogeneous cell wall suspension for agglutination studies, the cell wall suspension was stored at 3°C, without ever having been frozen or dried.

Agglutination of Cell Walls by Antisera.—The test system contained immunized rabbit antiserum (or normal serum), and purified cell walls in phosphate-buffered saline solution (0.15 M NaCl in 0.01 M phosphate, pH 7.3). For *qualitative studies*, 10 μl of the cell wall suspension (3.5 mg/ml) were added to 40 μl of serum at the proper dilution (containing hapten where appropriate) in small tubes (7 × 70 mm). This amount of cell wall provided a homogeneous translucent suspension. Hapten inhibition studies were performed by adding the suspected hapten to antiserum and preincubating at 37°C for 1 to 2 hours before addition of cell wall. Agglutination was detected as the appearance of granularity progressing to gross flocculation, with clearing of the supernatant in 10 to 15 minutes. No spontaneous agglutination was ever observed.

Agglutinations were followed with the aid of a hand lens, and the tubes were graded at frequent time intervals as follows: 0 = homogeneous milky suspension, 1+ = milky suspension with minimum granularity, 2+ = marked granularity, 3+ = cell walls clumped and partially settled, 4+ completely clumped, settled, and supernatant clear.

[1] Several antisera were also prepared with heat-killed or merthiolate-killed cells. Agglutination titers were similar to those of formalized cells, but these sera have not been investigated in detail.

For *quantitative studies*, 1 ml of buffered saline (containing hapten where appropriate) was added to 0.5 ml of undiluted antiserum (or undiluted normal serum), and incubated at 37°C for 1 hour. Then 1 ml containing 1.5 mg of cell wall in buffered saline was added and the incubation at 37°C was continued for another hour with frequent agitation. The tubes were then placed at 0–4°C for 12 hours. The suspension was centrifuged and the pellet, containing cell wall and adsorbed antibody, was washed 3 times in the cold with buffered saline. Protein in the pellet was then determined by the method of Lowry *et al.* (12) for insoluble proteins, using

TABLE I

Homologous and Heterologous Agglutination of Cell Walls of S. aureus, Strain Copenhagen

Agglutinations were read after 3 hours.

Antiserum prepared against:	Dilution of serum						
	Undiluted	1:5	1:10	1:20	1:40	1:80	1:160
S. aureus, strain Copenhagen	4+	4+	4+	4+	+4	2+	1+
Streptococcal Group A carbohydrate	4+	4+	1 to 2+	0	0	0	0
Horse serum azophenyl-β-acetylglucosaminide	4+	4+	1+	0	0	0	0
Normal rabbit serum	1+	0	0	0	0	0	0

TABLE II

Homologous and Heterologous Agglutination of Cell Walls of S. aureus, Strain Copenhagen, as a Function of Time

Agglutinations were carried out at the dilutions indicated.

Antiserum prepared against:	5 min.	15 min.	1 hr.	2 hrs.	4 hrs.
S. aureus, strain Copenhagen (1:40)	2+	4+	4+	4+	4+
Streptococcal Group A carbohydrate (1:5)	0	0	2+	3+	4+
Horse serum azoprotein (1:5)	0	0	1+	2+	3 to 4+
Normal rabbit serum (1:5)	0	0	0	0	0

the modified Folin-phenol reagent. This method completely solubilized cell wall and protein. A small amount of color in this determination was contributed by the cell wall itself, and was corrected for by subtracting a value obtained for cell wall in saline, instead of antiserum.

Haptens Related to the Cell Wall.—The cell wall of *S. aureus*, strain Copenhagen, is composed of two polymers, the glycopeptide (a polymer of acetylglucosamine and acetylmuramyl peptides which also contains a polyglycine component) and teichoic acid (a polymer containing ribitol phosphate, acetylglucosamine, and D-alanine). The two polymers are separated by 10 per cent trichloroacetic acid at elevated temperatures (*cf.* reference 1).

The following compounds were prepared by stepwise degradation of teichoic acid (7, 13): alanine-free teichoic acid, α-acetylglucosaminyl-polyribitol phosphate (the limit product of digestion of alanine-free teichoic acid by β-acetylglucosaminidase), glucosaminyl-ribitol phosphates and acetylglucosaminyl-ribitol phosphates (both of which are mixtures of the 1- and 2 phosphates and contain both α- and β-linked acetylglucosaminyl residues), glucosaminyl-ribitol and acetylglucosaminyl-ribitol.

The following compounds related to the glycopeptide were prepared by acid hydrolysis of the corresponding uridine diphosphate derivatives: acetylmuramic acid, acetylmuramyl-L-ala·D-glu·L-lys, and acetylmuramyl-L-ala·D-glu·L-lys·D-ala·D-ala (*cf.* reference 1). α-Methyl- and β-methyl-acetylmuramide were gifts from Dr. J. T. Park.

α- and β-phenyl-acetylglucosaminide were gifts from Dr. S. Roseman, and β-1,4-acetylglucosaminido-acetylglucosamine (diacetylchitobiose) was a gift from Dr. Luis Glaser. Strepto-

TABLE III

Hapten Inhibition of Homologous Agglutination of Cell Walls of S. Aureus, Strain Copenhagen

Haptens were preincubated with antiserum (at a dilution of 1:40) for 1 hour at 37°C, followed by addition of cell wall.

Hapten	Final concentration*	Degree of agglutination at:					
		5 min.	10 min.	15 min.	2 hrs.	8 hrs.	24 hrs.
	M						
None	—	2+	3 to 4+	4+	4+	4+	4+
Teichoic acid‡	0.0013	0	0	0	0	0 to 1+	3+
Alanine-free teichoic acid	0.0008	0	0	0	0	0	3+
α-acetylglucosaminyl- polyribitol phosphate	0.0003	0	0	0	0	0	0
Acetylglucosaminyl-ribitol phosphates	0.003	0	0	0	0 to 1+	1 to 2+	4+
Acetylglucosaminyl-ribitol	0.003	0	0	0	0 to 1+	1 to 2+	4+
Acetylglucosamine	0.01	0	0	0	1 to 2+	2+	3+
α-phenyl-acetylglucosaminide	0.0025	0	0	0	0	1 to 2+	3+
β-phenyl-acetylglucosaminide	0.0025	1 to 2+	3+	3 to 4+	4+	4+	4+
D-ribitol-5-phosphate§	0.01	2+	3 to 4+	4+	4+	4+	4+
Normal rabbit serum substituted for antiserum	—	0	0	0	0	0	0

* The indicated concentration of the immunologically active haptens is the lowest concentration which would produce complete inhibition when the agglutinations were read at 1 hour.

‡ This teichoic acid used was prepared from strain Copenhagen. Teichoic acids prepared from strains H or Duncan, in which essentially all of the acetylglucosamine residues are β-linked, were inactive as haptens. Polyribitol phosphate (prepared from these teichoic acids by treatment with dilute alkali and digestion with β-acetylglucosaminidase) was similarly inactive. Teichoic acid from strain 3528 in which essentially all of the acetylglucosamine residues are α-linked was active as a hapten, however (15).

§ The following compounds were also ineffective as inhibitors of agglutination at concentrations between 10^{-2} and 10^{-3} M: glucosaminyl-ribitol phosphates, glucosaminyl-ribitol, D-glucosamine, acetate, *N*-acetyl-D-galactosamine, β-1,4-acetylglucosamido-acetylglucosamine, bovine albumin azophenyl-β-acetylglucosaminide, streptococcal Group A carbohydrate, ribitol, D-ribose-5-phosphate, 1,4-anhydroribitol, acetylmuramic acid, acetylmuramic acid–peptides, L-alanine, α-methyl-acetylmuramide, β-methyl-acetylmuramide, D-alanine, DL-alanyl-DL-alanine, D-glutamic acid, L-lysine, glycine, glycylglycine, glycylglycylglycine, and glycylglycylglycylglycine.

coccal Group A carbohydrate and crystalline bovine plasma albumin azophenyl-β-acetylglucosaminide, as well as rabbit antisera prepared against the Group A carbohydrate and against horse serum azophenyl-β-acetylglucosaminide were gifts from Dr. M. McCarty. D-ribitol-5-phosphate and anhydroribitol were synthesized chemically (14). Sugars, amino acids, and peptides were obtained either from Mann Research Laboratories, New York, or from Sigma Chemical Co., St. Louis.

RESULTS

Agglutination of Cell Walls by Homologous Antiserum and Its Inhibition by Haptens.—The highest dilution of antiserum which produced a 4+ agglutination in 15 to 30 minutes was 1:40. Weaker agglutinations (1 to 2+) were observed at dilutions up to 1:160 and occasionally at 1:320 but these did not progress to 3+ or 4+ agglutinations even after 24 hours. In *undiluted* normal rabbit serum a 1 or 2+ agglutination was observed after 2 hours, but no agglutination could be detected in normal serum diluted even 1:2 (Tables I and II).[2] Cell walls of *Micrococcus lysodeikticus* were not agglutinated by antiserum and cell walls of *S. aureus*, strain Duncan, were only agglutinated at low titer (1:5).[3]

TABLE IV

Hapten Inhibition of Agglutination of Cell Walls of S. Aureus, Strain Copenhagen, by Antiserum Prepared against Streptococcal Group A Carbohydrate

Hapten	Final Concentration	Degree of agglutintion at:			
		1 hr.	2 hrs.	3 hrs.	4 hrs.
None	—	2+	3+	3+	4+
Teichoic acid	0.002 M	0	0	1+	2+
α-acetylglucosaminyl-polyribitol phosphate	0.004 M	2+	2+	3+	4+
Acetylglucosaminyl-ribitol phosphates	0.002 M	0	0	0	0
Glucosaminyl-ribitol phosphates	0.005 M	2+	2+	3+	4+
Acetylglucosamine	0.01 M	0	0	0	0
α-phenyl-acetylglucosaminide	0.0025 M	1 to 2+	2+	3+	4+
β-phenyl-acetylglucosaminide	0.0025 M	0	0	0	0
β-1,4-acetylglucosamido-acetylglucosamine	0.005 M	0	0	0	0
Streptococcal Group A carbohydrate	1 mg/ml	0	0	0	0
Bovine albumin azophenyl-β-acetylglucosaminide	1 mg/ml	1+	1 to 2+	2+	2 to 3+

[2] Most of the experiments reported have been carried out with serum from three immunized rabbits with similar results with each antiserum. Serum samples from four different normal rabbits have been employed as controls.

[3] The low titer agglutination of cell walls of strain Duncan is due to the presence of antibodies specific for β-linked acetylglucosamine residues (15). The teichoic acid of this strain (as that of strain H) contains essentially all β-linked acetylglucosamine residues, and induces

Hapten inhibition of agglutination was demonstrable only at a dilution of 1:40. At lower dilutions the strong and rapid agglutination observed was not inhibitable and at higher dilutions agglutination itself was too weak to be certain of its inhibition. Preincubation of haptens with antiserum at 37°C was essential, and with some antisera a better inhibition was observed if preincubation was carried out for 5 to 6 hours. No inhibition was observed if haptens and cell wall were added to antiserum sequentially without preincubation. Among the haptens tested only teichoic acid, alanine-free teichoic acid, α-acetyl-

TABLE V

Hapten Inhibition of Agglutination of Cell Walls of S. aureus, Strain Copenhagen, by Antisera Prepared against Horse Serum Azophenyl-β-acetylglucosaminide

Hapten	Final concentration	Degree of agglutination at:			
		1 hr.	2 hrs.	3 hrs.	4 hrs.
None	—	1+	2+	3+	3 to 4+
Teichoic acid	0.002 M	0	0	1+	1 to 2+
α-acetylglucosaminyl-polyribitol phosphate	0.004 M	1+	2+	3+	3 to 4+
Acetylglucosaminyl-ribitol phosphates	0.002 M	0	0	0	1+
Glucosaminyl-ribitol phosphates	0.005 M	1+	2+	3+	3 to 4+
Acetylglucosamine	0.01 M	0	0	0	0
α-phenyl-acetylglucosaminide	0.0025 M	1+	1+	2+	2+
β-phenyl-acetylglucosaminide	0.0025 M	0	0	0	0
β-1,4-acetylglucosaminido-acetylglucosamine	0.005 M	0	0	0	0
Streptococcal Group A carbohydrate	1 mg/ml	1+	2+	3+	3 to 4+
Bovine albumin azophenyl-β-acetylglucosaminide	1 mg/ml	0	0	0	0

glucosaminyl-polyribitol phosphate, acetylglucosaminyl-ribitol phosphates, acetylglucosaminyl-ribitol, acetylglucosamine, and α-phenyl-acetylglucosaminide were active (Table III).

Heterologous Agglutinations of Cell Walls and Its Inhibition by Haptens.— Antisera prepared against streptococcal Group A carbohydrate (in which a terminal β-linked acetylglucosamine residue is the immunological determininant, references 16 and 17) and against horse serum azophenyl-β-acetylglucosaminide both agglutinated cell walls of *S. aureus*, strain Copenhagen. In contrast to the homologous agglutination, however, the titers of these sera were low (1:5) and the agglutination required several hours for full development (Tables I and II).

Acetylglucosamine, β-phenyl-acetylglucosaminide, β-1,4-acetylglucosamin-

in rabbits antibodies specific for the β-configuration (15). No antibodies specific for the α-configuration are induced by strains Duncan or H, however. The formation of antibodies specific for the β-acetylglucosaminide unit in the teichoic acid has also been demonstrated with strain NYH-6 (10).

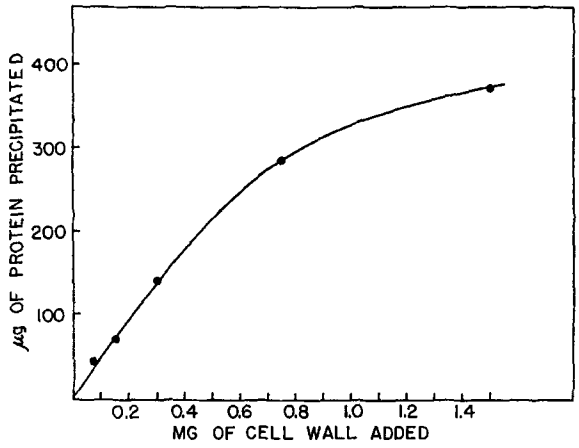

FIG. 1. Quantitative measurement of protein adsorbed by cell wall from antiserum.

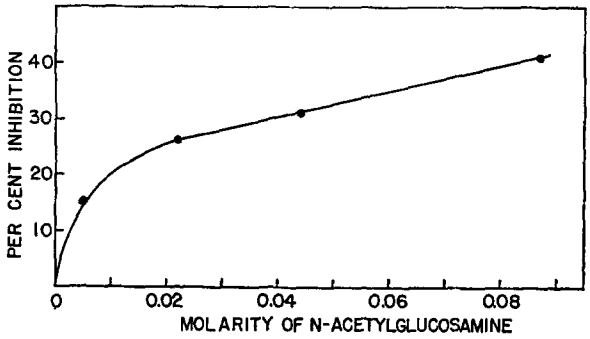

FIG. 2. Inhibition of adsorption of antibody by *N*-acetylglucosoamine.

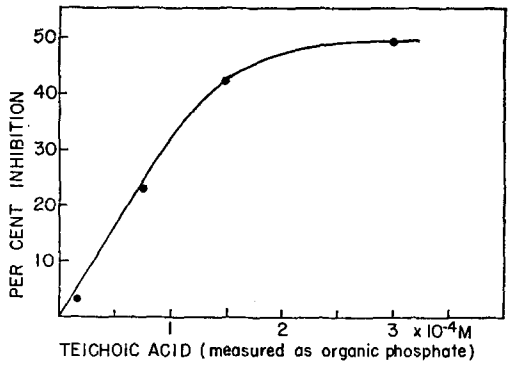

FIG. 3. Inhibition of adsorption of antibody by teichoic acid.

ido-acetylglucosamine, the homologous antigen and acetylglucosaminyl-ribitol phosphates inhibited both agglutinations. Teichoic acid also inhibited, but only partially at the concentration tested. In contrast to the homologous agglutination, neither of the heterologous agglutinations was inhibited by α-phenyl-acetylglucosaminide, or by α-acetylglucosaminyl-polyribitol phosphate (Tables IV and V)

Preliminary Quantitative Studies.—Limited quantitative data on the homologous agglutination and its inhibition by haptens has been obtained. These experiments were carried out at 50 times the scale of the qualitative experiments, and it has not been possible as yet to employ those haptens which were available only in small amounts.

With a constant amount of antiserum, the amount of antibody adsorbed to cell wall appeared to approach a plateau as the amount of cell wall increased (Fig. 1). No adsorption of protein from normal rabbit serum by these cell walls was detected. The maximum inhibition of antibody adsorption observed at high levels of acetylglucosamine (Fig. 2) or teichoic acid (Fig. 3) was 40 to 50 per cent of the protein adsorbed in the absence of hapten.

DISCUSSION

It is apparent from these studies that an α-acetylglucosaminyl-ribitol grouping in the teichoic acid is an immunologically active group in the cell wall of *S. aureus*, strain Copenhagen. The teichoic acid of this strain is a polymer composed predominantly of 4-0-β-*N*-acetyl-D-glucosaminyl-D-ribitol units linked by 1,5-phosphodiester bridges (7, 13). About 15 per cent of the acetylglucosaminyl-ribitol units have the α-configuration, however, and half of the ribitols are also esterified with D-alanine at the 2- or 3-hydroxyl. The average number of units in a polymer molecule is 12 to 16. The polymer can be degraded by removal of D-alanine (by dilute alkali) and of the β-linked acetylglucosamine residues (with a β-acetylglucosaminidase), leading to formation of a compound termed α-acetylglucosaminyl-polyribitol phosphate. Degradation of teichoic acid with strong alkali led to the isolation of acetylglucosaminyl-ribitol phosphates, which yielded acetylglucosaminyl-ribitol on treatment with a phosphatase. The teichoic acid from *S. aureus*, strain H (18, 19) is a similar, but not identical, substance. The amount of α-acetylglucosaminyl-ribitol units in the teichoic acid of this strain (1 to 5 per cent of the total) is insufficient to provoke in rabbits a significant titer of antibodies specific for the α-configuration (15).

The teichoic acid from the cell wall of strain Copenhagen and all degradation products of it which contained the α-acetylglucosaminyl-ribitol grouping (*e.g.* acetylglucosaminyl-ribitol itself) were immunologically active as haptens in inhibiting cell wall agglutination. All other degradation products of the teichoic acid (*e.g.* D-ribitol-5-phosphate) were inactive as haptens under the conditions employed, and small molecular weight substances related to the glycopeptide

were similarly inactive. α-Acetylglucosaminyl-polyribitol phosphates had equal or greater potency as haptens than teichoic acid. Similarly, α-phenyl-acetylglucosaminide and acetylglucosamine itself were immunologically active while several compounds which contained a β-linked acetylglucosamine residue were immunologically inactive. Inhibition by haptens thus clearly implicates the α-acetylglucosaminyl-ribitol unit as the major agglutinating antigen.

The heterologous agglutination of cell walls by two antisera with a specificity for the β-acetylglucosaminide linkage (antiserum against streptococcal Group A carbohydrate and antiserum against horse serum azophenyl-β-acetylglucosaminide, prepared by McCarty, references 16 and 17) showed that the β-acetylglucosaminyl-ribitol residues of the teichoic acid are available for reaction with appropriate antisera, although by the techniques employed in this experiment antibodies directed against these groups were not found in homologous rabbit antiserum. Although it was possible that these groups were incompetent as inducers of antibody, such β-acetylglucosaminide–specific antibodies are in fact present in these homologous rabbit antisera, but at low titer (15).

Hapten inhibition of cell wall agglutination could be demonstrated only at the highest dilution of antiserum which produced agglutination. Therefore, any antibody present at lower titer would not be demonstrable by the present techniques. It is possible, therefore, that lower titer antibodies formed against other linkages in the teichoic acid are also present in homologous rabbit antiserum. Moreover, antigens might also be present in the cell wall which are not agglutinogens, and hence could not be detected by these techniques under any circumstances. No evidence for immunological activity of any component of the glycopeptide was obtained but this possibility requires further investigation. In quantitative studies only 40 to 50 per cent of antibody adsorption to cell wall could be blocked by acetylglucosamine or by teichoic acid, suggesting that some antibody specific for other sites on the cell wall might also be present.

The complex immunological structure of *S. aureus* strains has been recently reviewed by Oeding (20). It is not possible at the present time to say which, if any, of the *S. aureus* factors (20) is the α-acetylglucosaminyl-ribitol determinant. In addition to the teichoic acids, several capsular substances (which do not appear to be teichoic acids) in unusual strains of *S. aureus* have been identified as immunologically active materials (21, 22).

SUMMARY

Antisera, prepared against formalin-killed cells of *Staphylococcus aureus*, strain Copenhagen, agglutinated the cell walls of this strain. The agglutination was inhibited by the teichoic acid from the cell wall of this strain, by any degradation product of this teichoic acid which contained the α-acetylglucosaminyl-ribitol unit, by α-phenyl-acetylglucosaminide, and by *N*-acetylglucosamine, but not by a large number of other haptens related to the cell wall.

In quantitative experiments, however, only 40 to 50 per cent of antibody adsorption to cell wall could be inhibited by teichoic acid or by *N*-acetylglucosamine. The α-acetylglucosaminyl-ribitol unit in the teichoic acid is, therefore, an important immunological determinant in the cell wall of this strain, although other immunological specificities may also exist.

The cell walls were also agglutinated by heterologous antisera prepared against streptococcal Group A carbohydrate or against horse serum azophenyl-β-acetylglucosaminide. The heterologous agglutination, however, was specific for the β-acetylglucosaminyl-ribitol units in the teichoic acid.

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