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Chemical Composition and Immunological Specificity of the Streptococcal Group O Cell Wall Polysaccharide Antigen

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The group O streptococcal group antigen was shown to be a polysaccharide located in the cell wall of the organism. The antigen could be extracted by one of several methods: (i) 0.5 N NaOH at 37 C, (ii) phenol-water (50:50) at 68 C, (iii) 0.2 N HCl at 100 C, or (iv) 10% trichloroacetic acid at 4 C. The last method yielded more polysaccharide with less protein contamination. The polysaccharide was purified on diethylaminoethyl-Sephadex A-25 and Sephadex G-200. It was composed of two-thirds glucosamine and galactosamine, and the remainder glucose plus galactose. Rhamnose, glycerol, ribitol, and muramic acid were absent. Total phosphorus and amino acids were each less than 0.1%. *N*-Acetyl-β-D-glucosamine exerted a strong inhibition of the precipitin reaction and is considered the immunodominant sugar. Glucosamine and glucose possessed a partial inhibitory activity. Galactose and galactosamine were essentially negative. No evidence of cross-reactivity was found between the O polysaccharide and group A and L polysaccharides, and group A and *Staphylococcus aureus* teichoic acids, which posesss *N*-acetylglucosamine specificity. The release of limited quantities of *N*-acetylglucosamine from its terminal location by enzyme, and glucose by acid hydrolysis, indicates a limited number of side chains in the O antigen. The glucosamine is in acid-stable linkage in the polysaccharide. Glucose was not released by β-glucosidase and probably does not occupy a terminal position. The O antigen is the only known streptococcal polysaccharide antigen which does not contain rhamnose. The effect of these factors on the immunological specificity is discussed. O serum, after adsorption with the purified polysaccharide, was used to demonstrate the presence of protein antigens in acid extracts of cells from each of the nine strains examined. These antigens may represent type antigens. Two of these strains, originally described as group O, did not contain the O polysaccharide.

The group O streptococci are alpha- and beta-hemolytic, lactose-fermenting organisms which can be isolated from the human throat (3). They cause pneumonia and subacute bacterial endocarditis in man (7, 8).

The group polysaccharide antigens of groups A (20), B (14, 33), C (13), E (27), F (32), G (5, 6), L (12), and R (28), and type antigens of groups D (2, 9) and F (21) all contain rhamnose. It appears likely from these results and the occurrence of rhamnose in the cell wall of most streptococcal groups (26) that rhamnose is an important constituent of the streptococcal antigen polysaccharides. The cell walls of four strains of group O streptococci, however, were found to contain glucose, galactose, glucosamine, and galactosamine but no rhamnose (26). Agglutination of the cell walls of these strains with group-specific serum indicated the presence of the group antigen in the cell wall (26).

It seems evident that the group O polysaccharide differs from the normal pattern. The present report presents data on the extraction, composition, and immunological specificity of the antigen.

MATERIALS AND METHODS

Streptococcal strains. We are grateful to W. R. Maxted, R. C. Lancefield, R. R. Facklam, C. E. de Moor, and H. W. B. Engel for the group O strains used. Each was reisolated on sheep blood agar and tested with group O-specific antiserum by the capillary precipitin test (29). The strains used were B357, B361, 1360, 11019, SS533, SS669, SS808, SS809, and DS-1234. The cells for study were grown, harvested, and lyophilized as previously described (10, 26). We thank

Aaron Lane, Difco Laboratories, Detroit, Mich., for some of the B357 cells.

Antisera. B357 cells were used to immunize New Zealand Red rabbits (26). The sera were adsorbed with streptococcal cells of a group (group A) other than O as described earlier (27). Other group O sera were kindly provided by the Center for Disease Control, Atlanta, Ga., and C. E. de Moor and H. W. B. Engel, Utrecht, Holland.

Preparation of crude antigen. Crude antigen extracts were obtained by the following three methods. (i) Lyophilized whole cells (50 mg) of each group O strain (B357, B361, 1360, and 11019) were extracted with 1 ml of 0.2 N HCl at 100 C for 10 min. The cells were removed by centrifugation, and the supernatant fluids were adjusted to *p*H 7.4 with 0.2 N NaOH. This preparation is labeled WCa in Fig. 1. (ii) Whole cells (50 mg) were extracted with 2 ml of 10% trichloroacetic acid at 4 C with continuous stirring overnight. The cells were removed by centrifugation, and the supernatant fluids were dialyzed against distilled water and saline. This preparation is labeled WCt in Fig. 2. (iii) Cell walls (20 mg) prepared as previously described (25) were extracted with 10% trichloroacetic acid at 4 C as described above. These crude preparations were used to determine the efficiency of the extraction procedure, the number of antigens extracted in each case, and the location in the cell of the antigen.

Purification of antigen. Two procedures were used. (i) A 30-g amount of lyophilized whole cells (strain B357) was extracted with 500 ml of 10% trichloroacetic acid as above. The extraction was repeated twice with 300 ml of trichloroacetic acid. The extracts were combined, dialyzed four times overnight at 4 C in 5 liters of distilled water, flash-evaporated, and lyophilized. The yield of nondialyzable material was 240 mg. The material was applied to a column (1.5 by 50 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 (HCO_3^- form). The material was eluted with a linear concentration gradient of 330 ml of 0.5 M $(NH_4)_2CO_3$, *p*H 8.6, and 330 ml of 0.25 M $(NH_4)_2CO_3$. Each fraction (5 ml) was collected and analyzed. The fractions which showed a positive precipitin test with specific group O antiserum were combined (total dry weight 25.0 mg) and further purified with Sephadex G-200 in 0.05 M $(NH_4)_2CO_3$, *p*H 8.6. The yield was 11.5 mg. This material (Flt) was used for chemical analysis of the polysaccharide (Table 1). (ii) A 4-g amount of cell wall was extracted with 200 ml of 10% trichloroacetic acid at 4 C overnight, and the extraction was twice repeated with 100 ml of 10% trichloroacetic acid. Three volumes of ethanol were added to the combined extracts, and the precipitate was removed by centrifugation, and dried under a vacuum. The yield was 10.8 mg. The material was applied to a DEAE-Sephadex A-25 column and then to a Sephadex G-200 column as described above. The yield was 3.35 mg.

Analytical methods. The polysaccharide preparations were hydrolyzed in 4 N HCl at 100 C for 12 hr and then examined for reducing sugars, amino acids, and glycerol after separation by thin-layer chromatography (16). Total phosphorus was measured as previously described (16).

Sugars were determined quantitatively by liquid-gas chromatography (4). The dry sample (10 to 50 μg) was methanolized with 0.5 ml of a solution consisting of 0.5 N HCl in absolute methanol in a sealed tube overnight. The sample was dried and acetylated in a sealed tube containing 0.5 ml of acetylation reagent (7.5 ml of absolute methanol + 2.5 ml of acetic anhydride + 1 mg of silver acetate) overnight at room temperature. After drying in a vacuum at room temperature the material was treated with 10 to 100 μliters of silation reagent (pyridine-hexamethyldisilazane-trimethylchlorosilane, 5:1:1) at room temperature for 10 min. One to 10 μliters of the solution was injected into a Varian Aerograph model 1200 gas chromatograph. The glass column (3 mm by 1.8 m) was packed with 5% SE-30-coated chromasorb W (60/80 mesh). Sensitivity and chart speed were 1 × 16 and 0.83 cm/min, respectively. The operating temperature was 160 C for the first 15 min and then increased at the rate of 4 C/min to 200 C. Two-tenths to 2.0 μg of authentic sugars were used as standards. Amino sugars were analyzed on a Spinco amino acid analyzer as previously described (16).

Serological procedure. Agar-diffusion analysis of the antigen-antibody complex was carried out as described by Ouchterlony (23). The qualitative and quantitative precipitin reactions, and the inhibition of the latter, have been described (16).

Lability of antigen. A 10-μg amount of antigen was treated with 20 μliters of 0.05 N to 2 N HCl at 50 to 100 C. After several minutes, the solution was neutralized with NaOH and the volume was made to 25 μliters. The quantitative precipitin determination was performed on the neutralized solution.

Enzyme activity on the antigen was tested using 1% (w/w) β-glucosidase (Worthington Biochemical Co., Freehold, N.J.) in 0.01 M phosphate buffer (*p*H 6.0), 1% lysozyme (Worthington Biochemical Co.), or 1% trypsin (Mann Research Labs., New York, N.Y.) in 0.01 M phosphate buffer (*p*H 7.4), and 1% pepsin (General Biochemicals Corp., Cleveland, Ohio) in HCl (*p*H 2.0) for several hours.

Release of sugars. A 75-μg amount of group O polysaccharide antigen was treated with 75 μliters of 0.1 N HCl at 100 C for 1 to 3 hr. After being neutralized with 0.1 N NaOH, the reaction mixtures were separated into high-molecular-weight fractions (tubes no. 10–15) and low-molecular weight fractions (tubes no. 23–30) on a Sephadex G-15 column (1 by 55 cm) in distilled water; 0.89 ml was collected in all cases. The lower-molecular-weight fractions were lyophilized and analyzed by gas chromatography as described above.

N-Acetyl-β-D-glucosaminidase (from bovine aorta) was used at *p*H 4.4 for up to 12 hr with 30 μg of polysaccharide (Flt). We wish to thank Georg Springer for the enzyme.

RESULTS

The first extracts of the group O strains were made with 0.2 N HCl at 100 C for 10 min (29). Lyophilized whole cells of strains 1360, B357, B361, and 11019 were used. It can be seen in Fig. 1 that each of the HCl extracts contained one or more antigens which reacted with B357 antiserum.

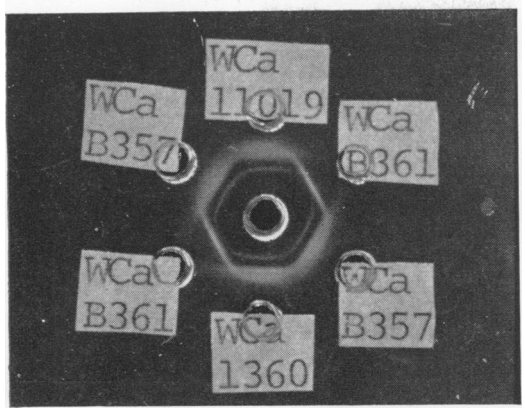

FIG. 1. *Reaction of HCl with group O serum. A 15-μliter amount of whole-cell extract containing material from 0.38 mg of cells in outer wells. Center well contained 10 μliters of anti-O (B357) serum. WCa: HCl extract of whole cells.*

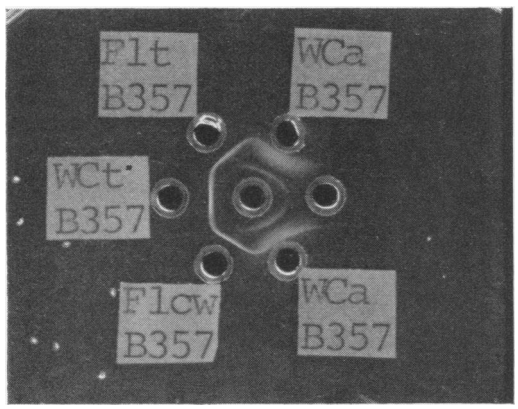

FIG. 2. *Reaction of whole-cell extracts and purified antigen with group O antiserum. WCa: same as Fig. 1; WCt: material from 0.38 mg of dry whole cells extracted by 10% trichloroacetic acid at 4 C for 18 hr; Flt: material from WCt after passage through Sephadex as described in Materials and Methods, 15 μg in well; Flcw: chromatographed material (15 μg) from cold trichloroacetic acid cell wall extracts. Fifteen μliters in each outer well and 10 μliters of B357 antiserum in inner well.*

The antigen common to all strains was most likely the group O antigen. Additional methods of extraction, 0.5 N NaOH at 37 C for 4 hr (1) and phenol-water (50:50 v/v) at 68 for 1 hr (15), were used, and the extracts were tested for the O antigen by the capillary precipitin test (29). Neither of these methods extracted sufficient antigen to be considered for large-scale work.

The method found suitable employed 10% trichloroacetic acid at 4 C. The yield was several-fold greater than the other procedures investigated, and it can be seen in Fig. 2 (WCt) that a single antigen was present as compared to at least three antigens in the HCl extract (Fig. 1, WCa B357). This single antigen was present in the largest quantity in the HCl extract of each of the four strains (Fig. 1).

Figure 2 also shows that the antigen extracted from whole cells by cold trichloroacetic acid (WCt) possessed a reaction of identity with the antigen extracted from cell walls (Flcw) by the same procedure. The results indicate that the principal location of the group O antigen is in the cell wall.

Figure 3a shows the chromatographic purification of the trichloroacetic acid extract of B357 cell walls. It is evident that the DEAE-Sephadex A-25 column achieved an excellent separation of the bulk of the contaminating protein when eluted

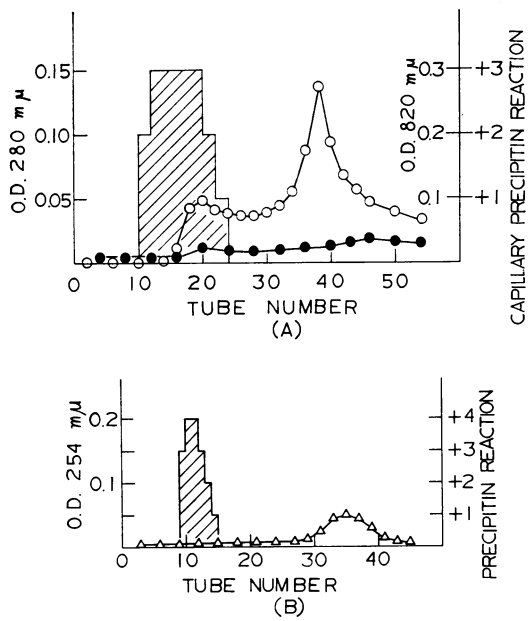

FIG. 3. *(A) Purification of group O antigen from strain B357 on DEAE-Sephadex A-25 column. The 10% cold trichloroacetic acid extract of cell walls was dialyzed against distilled water, lyophilized, and added to the column. Column length, 1.5 by 50 cm; starting buffer, 330 ml 0.05 M $(NH_4)_2CO_3$; final buffer, 330 ml 0.25 M $(NH_4)_2CO_3$; each tube, 5 ml. Protein (280 nm), ○; phosphorus (820 nm), ●; shaded area, precipitin reaction against group O (B357) antiserum. (B) Elution of group O polysaccharide antigen on Sephadex G-200 column. The fractions shown in Fig. 1 were collected, lyophilized, and added to the column. Buffer, 0.05 M $(NH_4)_2CO_3$, pH 8.6; column length, 1 by 55 cm; each tube, 1.55 ml. Absorbance at 254 nm, △; shaded area, precipitin reaction against group O (B357) antiserum.*

with $(NH_4)_2CO_3$ buffers. The phosphate values were low. Those tubes containing antigen were combined and passed through Sephadex G-200 in carbonate buffer, *p*H 8.6 (Fig. 3b). Adsorption values at 254 nm indicate that the protein present in Fig. 3a has been removed, and that no significant contamination by nucleic acid or nucleoprotein, or both, is present. Those tubes containing antigen were lyophilized.

Preparation Flt (Fig. 2 and 3) was used for chemical analysis. Table 1 shows that the antigen is a polysaccharide and that the amino sugars, glucosamine and galactosamine, made up two-thirds of the total. Glucose and galactose accounted for the remainder. The absence of glycerol, ribitol, and muramic acid, and less than 0.1% phosphorus and amino acids show that the preparation was not contaminated by peptidoglycan, protein, or teichoic acids.

Figure 4 shows the quantitative precipitin curve of chromatographed preparations from an HCl whole-cell extract and a trichloroacetic acid cell wall extract against B357 antiserum. In each case the equivalence point was reached with approximately 10 μg of antigen. Each of the hexoses present in the antigen was tested for its ability to inhibit the precipitin test. It can be seen (Fig. 5) that *N*-acetyl-D-glucosamine was responsible for a strong inhibition (63%), and that D-glucosamine and D-glucose inhibited between 30 and 40% at a level of 10 μmoles. The three galactose sugars inhibited 15% or less. It appears from these results that *N*-acetyl-β-D-glucosamine occupies a terminal position in the side chain of the polysaccharide and is primarily responsible for its immunological specificity. D-Glucose may be the penultimate hexose, and as such possesses a significant inhibitory activity (Fig. 5). The difference between *N*-acetyl-β-D-glucosamine and β-D-glucosamine (Fig. 5) indicates that the acetyl group is a significant factor in the total immunological specificity of the amino sugar.

TABLE 1. *Chemical composition of group O polysaccharide antigen*

Component[a]	Per cent
Glucose	7.4
Galactose	15.4
Glucosamine	22.4
Galactosamine	42.5
Recovery = 87.7%	

[a] Fucose, ribose, sorbitol, arabinose, xylose, mannose, galacturonic acid, glucuronic acid, 2-deoxyribose, 2-deoxyglucose, glycerol, ribitol, rhamnose, and muramic acid were not present; total phosphorus and amino acids were each less than 0.1%.

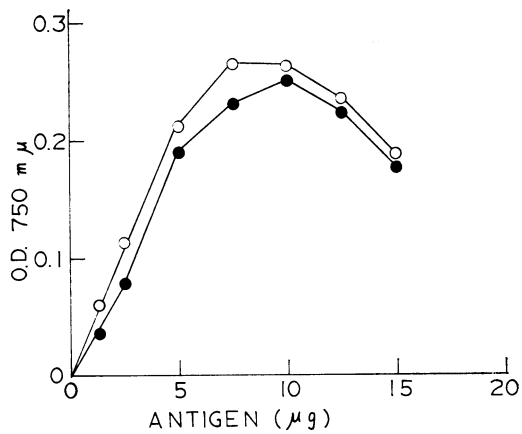

FIG. 4. *Quantitative precipitin curves of group O carbohydrate antigen against B357 antiserum. For each experiment 25 μliters of antigen solution and 25 μliters of serum were used. Purified polysaccharide antigen (Flcw) from whole HCl extract, ○; purified polysaccharide antigen (Flt) from 10% cold trichloroacetic acid cell wall extract, ●.*

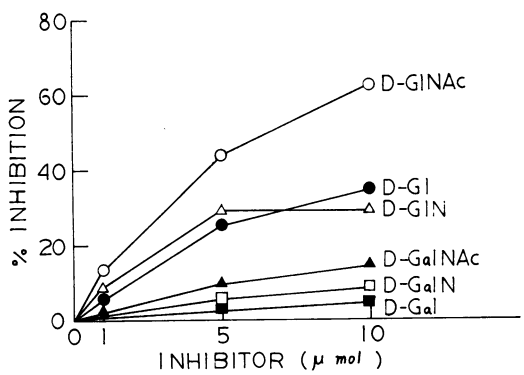

FIG. 5. *Inhibition of precipitin reaction between antigen Flt and B357 antiserum. To 25 μliters of antiserum, 25 μliters (10 μg) of antigen was added. Abbreviations: D-GlN Ac, N-acetyl-β-D-glucosamine; D-Gl, D-glucose; D-GlN, β-D-glucosamine; D-GalNAc, N-acetyl-β-D-galactosamine; D-GalN, β-D-galactosamine; D-Gal, D-galactose. The quantity of antigen and antibody used were at the equivalence point (cf. Fig. 4).*

The terminal position of *N*-acetylglucosamine adjacent to glucose is also supported by the data in Fig. 6. After 1 hr of hydrolysis in 0.1 N HCl at 100 C, the release of the glucosamine was complete (8.3% of the total); however, the free glucose amounted to about one-third of the total released in 3 hr, and the precipitin reaction remained positive. Both galactose and galactosamine were considerably less sensitive to the hydrolysis. After 3 hr only about one-third (as compared to glucosamine) had been released. After 2 hr of hydrol-

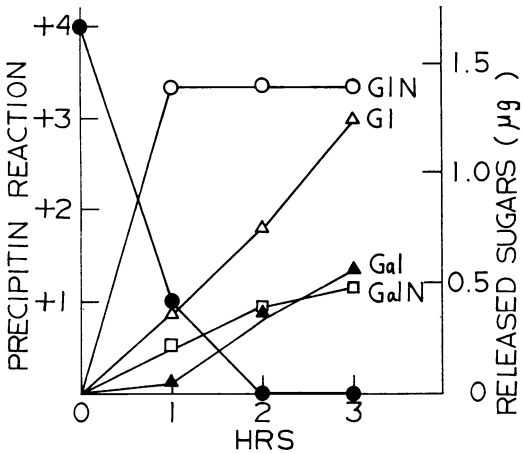

FIG. 6. *Release of sugars from 75 μg of group O polysaccharide antigen (Flt) by hydrolysis in 0.1 N HCl at 100 C. Sugars released were separated from the polymer on a Sephadex G-15 column (1 by 30 cm). Methylation, acetylation, and silation procedures were applied to samples of the chromatographed material, and the derivations were analyzed by gas chromatography. The glass column was packed with 5% SE-30-coated chromosorb W (60/80 mesh); sensitivity, 1 × 16; chart speed, 0.75 cm/min; operation temperature, 160 C for first 15 min and then increased at the rate of 4 C/min. Capillary precipitin reaction; ●, GlN, glucosamine plus N-acetyl glucosamine; Gl, glucose; Gal, galactose; GalN, galactosamine plus N-acetyl galactosamine.*

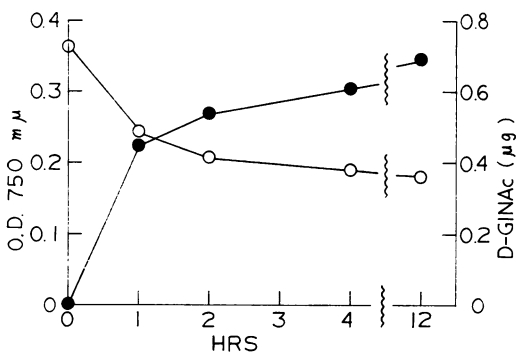

FIG. 7. *Release of terminal sugar from the polysaccharide antigen with N-acetyl-β-D-glucosaminidase. A 30-μliter amount of N-acetyl-D-glucosaminidase (0.05 μg/μliter) was added to 30 μliters of the antigen (30 μg) in 0.01 M citrate buffer, pH 4.4, and incubated for 1 to 12 hr at 37 C in the presence of 0.5% toluene. The reaction mixtures were passed through a Sephadex G-15 column (1 by 40 cm) in water, and the low-molecular-weight fractions were collected and dried in a vacuum and then analyzed by a gas chromatographic method. Quantitative precipitin reaction against group O antiserum: ○. For each experiment 30 μliters of reaction mixture and 30 μliters of serum were used. N-acetyl-β-D-glucosamine released: ●. Thin-layer chromatography showed that no other reducing sugar was released by the enzyme.*

ysis the antigen gave a negative precipitin reaction; 5% of the antigen by weight was released during the 3-hr hydrolysis.

Preparation Flt was tested with β-glucosidase to determine the possibility that glucose occupied a terminal position on some of the side chains of the antigen. After 6 hr at 37 C no glucose was released. The release of glucose from the control during this time was proof of enzyme activity. The results indicate that very little if any of the glucose in the antigen occupied a terminal position.

The polysaccharide (Flt) was also treated with *N*-acetyl-β-D-glucosaminidase to determine release of the amino sugar and loss of immunological activity. Figure 7 shows that the intensity of the precipitin reaction was reduced about one-half during a 12-hr exposure to the enzyme preparation, and that 5.4% of the total glucosamine was released. Group A polysaccharide from strain C203 (prepared from whole cells by trichloroacetic acid extraction) (24) was used as a control. The release of 18.2% of the total glucosamine proved the activity of the enzyme preparation.

Three different antisera were used to determine the presence of group O antibodies in this study. Two had been prepared in different laboratories from whole-cell vaccines of strain B357, and the third from DS545. HCl extracts of each of the nine strains available were positive by capillary precipitin test to each of the three sera. Strains SS533 and 808, however, contained much less of the antigens than the other strains. Agar diffusion assays were then made, using the strongest of the sera (B357, prepared in our laboratories). Figure 8 shows that DS1234 and SS669, however, contained very little if any of the polysaccharide antigen. Each of the other seven strains, however, showed a reaction of identity with the polysaccharide preparation from B357 (Flt). The positive precipitin reaction in DS1234 and SS669 was probably due to other antigens present in the HCl extracts.

DISCUSSION

The present results show that the specificity of the group O polysaccharide is dependent, to a significant degree, on *N*-acetyl-β-D-glucosamine. The group-specific polysaccharide antigens of streptococcal groups A (20) and L (12), the group A teichoic acid (16), and a cell wall polysaccharide of *Streptococcus bovis* (an undesignated antigen) (11) likewise depend to a large extent for their specificity on *N*-acetyl-β-D-glucosamine. This common specificity is responsible for cross-reactivity between the A and L polysaccharides, and *Staphylococcus aureus* teichoic acid (12). Group O polysaccharide antiserum, however, does not react with these antigens or the group A teichoic

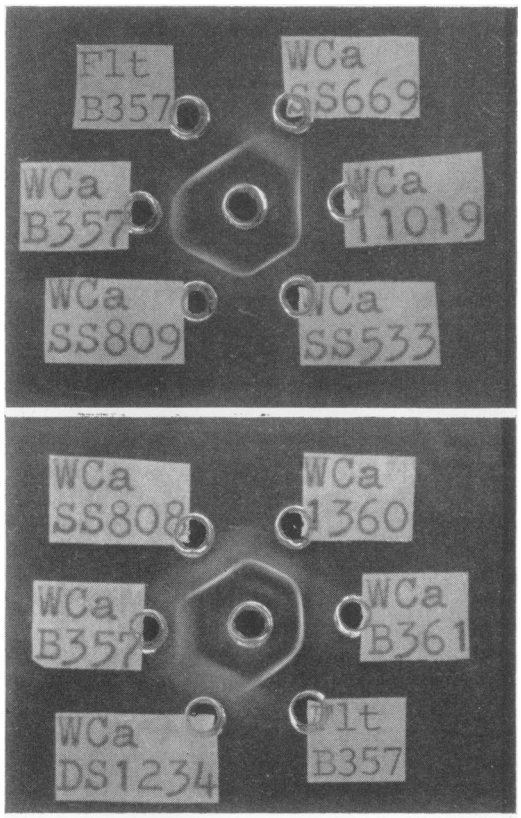

FIG. 8. *Agar-gel diffusion test of HCl extracts of group O strains and purified polysaccharide antigen (Flt) against B357 serum. Cells from 5-ml cultures (SS533 and SS808 from 25 ml) were extracted for 10 min with 1 ml of 0.2* N *HCl at 100 C, and the supernatant fluids were neutralized with 0.2* N *NaOH. Center well contained 20 μliters of anti-O serum and each outer well contained 20 μliters of the HCl extracts (WCa) or 20 μg of polysaccharide antigen (Flt).*

acid (16), and neither do these sera react with the O antigen. The *S. bovis* antigen and serum were not available for testing. On the other hand, the specificity of a type polysaccharide antigen of a type B streptococcus did not depend on *N*-acetylglucosamine, although the antigen contained 31% of the amino sugar. The terminal location of the sugar was not established however (31).

The release of glucosamine from the A, L, and O polysaccharides by *N*-acetyl-β-D-glucosaminidase is evidence of a terminal location of the glucosamine in each polysaccharide. The number of such enzyme-labile glucosaminyl residues in the O antigen, however, is only about one-third that of the A and L antigens. The failure of the O polysaccharide to cross-react with either the A or L antigen indicates the absence of a common specificity based in part on the terminal location of the amino sugar. It appears possible that the structure of the antigenic site in the O polysaccharide differs markedly from that in the A and L antigen.

Data in addition to those shown in Fig. 6 show that the *N*-acetyl-β-D-glucosaminyl linkage in the O antigen exists in a linkage stable to weak acid. HCl (0.05 N) at 100 C for 60 min caused no change in the precipitin reaction. Hydrolysis of the L antigen in 0.01 N HCl at 100 C for 5 min resulted in complete loss of glucosamine and serological activity. The stability of the O antigen to 0.05 N HCl also indicates the absence of phosphodiester groups, in agreement with the absence of phosphorus in the polymer (Table 1). Galactose and galactosamine were also resistant to hydrolysis; however 27% of the total glucose was released (Fig. 6). The inability of β-glucosidase to release glucose indicates that side chains containing β-glucose, if they exist, are protected against action of the enzyme.

It has been pointed out (26) that the specificity of the great number of streptococcal polysaccharide group and type antigens, many of which have not as yet been identified and described, would probably depend to some degree on the nature of the subterminal hexose. The limited data available seem to indicate that such is the case. The A and L polysaccharides possess rhamnose as the penultimate sugar and do not contain glucose (12, 20). On the other hand, glucose probably occupies the penultimate position in the O polysaccharide. The presence of rhamnose in all the streptococcal group polysaccharides except O may help to explain further the specificity of the latter. The specificity of the E and F polysaccharides, although both possess a terminal β-D-glucose, is dependent to a great extent on the nature of the penultimate sugar (27, 32). Other variables which may be involved in specificity are length of side chains, linkage between sugars, and alpha or beta forms (26). The latter forms of *N*-acetyl-D-glucosamine in *S. aureus* teichoic acid are each responsible for a specificity (30). Table 1 shows that both the sugar and amino sugar present in the polysaccharide in smallest quantity, compared to others in the same antigen, possess the prinicipal specificity. It appears that the specificity of the O polysaccharide is shared between *N*-acetyl-β-D-glucosamine and β-D-glucose, and that other undefined structural features of the antigen are also involved.

The positive precipitin and negative gel diffusion reactions for strains DS1234 and SS669 indicate that other antigens, present in the acid extracts and specific for the group O streptococci, and not a part of the isolated group polysaccharide, are responsible for the difference. Figure 1

shows that crude acid extracts contain several antigens in addition to the polysaccharide. Adsorption of our O sera with the O polysaccharide (Flt) did not remove these antigens, and the precipitin test remained positive for all strains. Preliminary studies indicate that these are proteins and may represent a type antigen. It seems likely that strains DS1234 and SS669, which do not contain the O polysaccharide, contain a type antigen(s) which is common to most O strains. They do not react to streptococcal group A, B, C, D, E, F, G, H, K, L, M, N, O, P, Q, R, and S antisera; however they probably contain an unidentified polysaccharide antigen. The F polysaccharide type antigens are found in streptococci possessing the group C, D, G, and T antigens (22), and the M, R, and T protein antigens of group A are found in B, C, and G cells (17–19).

Our experience with the streptococci has demonstrated that dilute trichloroacetic acid, at either high or low temperature, is an effective solvent for removing cell wall polysaccharide (24). Hydrolysis of the antigen by trichloroacetic acid has not been encountered; however the usual procedure using 0.05 N HCl at 100 C will destroy the R antigen (28). Another advantage of trichloroacetic acid is the small quantity of contaminating protein present in the extract (Fig. 2). Purification of the polysaccharide is facilitated, and the possibility of false-positive precipitin reactions is reduced.

ACKNOWLEDGMENTS

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