THE PRESENCE OF A GROUP A VARIANT-LIKE ANTIGEN IN STREPTOCOCCI OF OTHER GROUPS WITH SPECIAL REFERENCE TO GROUP N

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Immunological relationships between the type-specific antigens of certain strains of Group D and Group N streptococci have been described (1). The Group D (enterococcal) type antigens are cell wall carbohydrates (2) and it seemed reasonable to suppose that in Group N also, the type-specific substance would be located in the bacterial cell wall. In both Group D and Group N, the group-specific substances are chemically and serologically distinct polyglycerophosphates (teichoic acids) located, for the most part, deep in the bacterial cell (3, 4).

The initial purpose of the work described here was to determine the location and nature of the type-specific substances in two strains of Group N streptococci but during the course of the investigation an additional point of interest emerged. It was found that in the two strains of Group N cocci examined, the cell wall carbohydrates were immunologically related to the cell wall carbohydrate of the Group A-variant streptococcus described by McCarty and Lancefield (5). The latter has since been detected in some variant strains derived from Group C (6, 7). It will be shown here that an immunologically related carbohydrate occurs not only in the walls of Group N streptococci but also in streptococci belonging to Groups D, E, G, and M.

Materials and Methods

Streptococcal Strains and Antisera.—All strains and antisera used in this work were provided by Dr. R. C. Lancefield from her collection at The Rockefeller University. GpN strains C559 and B209 have been previously described (4). Strain C559 is sometimes designated the "Orla Jensen strain" or "strain 6681" in the National Collection of Industrial Bacteria. London, England. Formalin-killed vaccines were used in the preparation of rabbit antisera.

Precipitin Tests.—These were carried out using the capillary technique (8).

Quantitative Precipitin-Inhibition Determinations.—The optimal antigen–antibody ratio was first determined for cell wall lysozyme lysates of known concentration and the corresponding type-specific rabbit antisera. To each of a series of tubes was added first a known weight of cell wall antigen dissolved in 0.9 ml of saline and then 0.1 ml of an appropriate standard dilution of antiserum. The precipitate that formed during the ensuing 24 hr was

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washed three times in chilled saline (1 ml). It was then dissolved in 1 ml of 0.1 N NaOH and the protein content determined by the modified Folin and Ciocalteau method using Cohn's Fraction II as a standard. A Technicon auto analyzer (Technicon Corp., Ardsley, N.Y.) adapted to perform the Lowry determination (9) was used. With that amount of antigen which gave maximum antibody precipitation, the procedure was then repeated with 50 mg of inhibitor or control substance added to each tube. The percentage of inhibition was calculated by comparing the amount of gamma globulin precipitated in each tube with the amount precipitated in the control tube.

Preparation of Streptococcal Cell Walls from 5 Liters of Culture.—The streptococci were grown in Todd–Hewitt broth for 18 hr, harvested on the centrifuge, and resuspended in approximately 100 ml of distilled H_2O. They were then washed three times in this volume of water and finally suspended in approximately 50 ml. of H_2O. The washed coccal suspension was disrupted by shaking with glass beads (Ballotini No. 13) in a Braun mechanical disintegrator (H. Braun Tool & Instrument Co., Inc., Hawthorne, N.J.) with periodic, intercurrent cooling by liquid CO_2. The bacterial walls so obtained were checked for purity by microscopic examination which revealed no intact cocci in the final suspension. Glass beads were removed by passage through a coarse sintered glass filter. Using a high-speed centrifuge (13,000 g) the walls were then washed three times with saline and twice with phosphate buffer (pH 7.4) containing NaCl and $MgCl_2$ (buffer No. 1).[1] The washed walls were suspended in 5 ml of the buffer to which had been added RNAse and DNAse to a final concentration of 1.0 and 2.0 mg/ml respectively. The mixture was dialyzed at 37° C overnight in 4 liters of buffer No. 1 and then transferred to 4 liters of phosphate buffer No. 2 (pH 8.1) containing 9 g of NaCl. After allowing a short period for adjustment to the new buffer, crystalline trypsin was added to the wall suspension to a final concentration of 1 mg/ml and dialysis continued at 37° C for an additional 18 hr. The streptococcal walls were then washed three times in saline, three times in distilled water, and the final suspension was lyophilized.

Streptococcal Extracts.—Acid extracts were prepared as described by Lancefield (10).

Analytical Methods.—A preliminary qualitative analysis for monosaccharide cell wall components was made by paper chromatography after hydrolysis of the cell walls in 4 N HCl at 100°C for 6 hr under nitrogen in sealed ampoules. In the chromatographic procedures, the solvent was a mixture of butanol, pyridine and water (3:2:1.5) and the spraying agents were aniline hydrogen phthalate or ninhydrin.

Amino acids and amino sugars in acid hydrolysates were determined by ion-exchange chromatography with a Spinco automatic amino acid analyzer. Samples (about 1.0 mg) of the carbohydrates in cell wall lysates which had been passed through a column of Sephadex G-25 were hydrolyzed with 1.0 ml of 4 N HCl at 100°C in sealed, evacuated glass tubes for 6, 12, and 24 hr. The hydrolysates were evaporated to dryness in a rotary evaporator at 40°C and the residues were dissolved in 3.0 ml of the pH 2.2 buffer used with the amino acid analyzer. The solutions were filtered through a Millipore membrane (Millipore filter Corp., Bedford, Mass.) and 1.0 ml portions were analyzed. The values for the stable amino acids lysine, aspartic acid, glutamic acid, and alanine are the average of three determinations. The values for glucosamine, galactosamine, and muramic acid phosphate are extrapolated values to zero time using the values obtained from the 6, 12, and 24 hr hydrolysis.

Galactose was estimated by the Galactostat reagent (Worthington Biochemical Corp. Freehold, N.J.). Rhamnose was determined without prior hydrolysis using the cysteine–sulphuric acid method of Dische and Shettles (11). Phosphorus was determined by the method of Chen, Toribara, and Warner (12).

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[1] Buffer No. 1 contained Na_2HPO_4 (M.15) 500 ml, KH_2PO_4 (M. 15) 100 ml, NaCl 9 g, $MgCl_26H_2O$ 4 g, and H_2O to 4 liters. Buffer No. 2 contained Na_2HPO_4 (M.15) 500 ml, NaCl 9 g, and H_2O to 4 liters.

Electrophoretic Methods.—Preparative zone electrophoresis was performed in veronal buffer 0.05 м, pH 8.6 at 400 v with Pevikon as a supporting medium. Immunoelectrophoretic analysis was performed in veronal buffer 0.05 м, pH 8.6. The supporting medium was 1% Noble agar (Difco Laboratories; Inc., Detroit, Mich.) in 0.25 м veronal buffer on microscope slides (3 × 1 inch) employing 5 ma per slide.

RESULTS

Chemical Composition of Group N Streptococcal Cell Walls.—Table I presents the results of chemical analyses of cell walls from two strains of Group N

TABLE I

Chemical Composition of Cell Walls from Group N Streptococci, Strains C559 and B209

Cell wall component	Strain C559	Strain B209
	weight per 100 mg sample	weight per 100 mg sample
	(mg)	(mg)
Rhamnose	35.2	23.9
N-acetylglucosamine	12.1	21.4
N-acetylgalactosamine	5.97	0
Galactose	0	3.0
Muramic acid phosphate	2.77	4.14
Lysine	2.62	2.63
Aspartic acid	0.85	1.05
Glutamic acid	4.40	6.29
Alanine	2.20	3.11
Phosphorus	1.3	0.6
Weight recovery*	66.11	65.2
Nitrogen recovery‡	55.1	39.0

* The weight recovery does not include moisture and ash, which usually account for 14–16% of the sample. Phosphorus was not included in the weight recovery except as a component of muramic acid phosphate.

‡ Nitrogen recovery includes glucosamine, galactosamine, muramic acid phosphate, and the amino acids listed, but not ammonia.

streptococci, C559 and B209. The streptococcal walls, prepared as described under Materials and Methods, were protein-free, but it will be recalled that digestion with trypsin was part of the procedure used in their preparation. Although the carbohydrates so far identified account for not more than 66% of the dry weight, most of the cell wall serological activity to be described is referable to the sugar components listed.

Precipitin Reactions with Lysed Cell Walls from Group N Streptococci.—10 mg amounts of lyophilized streptococcal walls prepared from cultures grown either at 37°C or at room temperature were suspended in 1 ml quantities of water containing crystalline lysozyme in a concentration of 1 mg/ml. Almost complete lysis occurred during subsequent incubation of the mixtures at 37°C

for 6 hr. In this way approximately 1% (w/v) solutions of cell wall material were prepared from strains C559 and B209.

From each 1% cell wall lysate, three serial 10-fold dilutions in saline were prepared and tested by the capillary precipitin method (8) with rabbit antisera of homologous and heterologous strain and group. The results of these tests are set out in Table II.

Type-specific reactions: It will be noted that cell wall preparations reacted at concentrations of 0.01 mg/ml with homologous antisera, but reacted with heterologous antisera containing Group N antibody only when tested in concentrations of 1 mg or more per ml. Group reactions of this kind occurred only with cell walls prepared from cultures grown at room temperature and

TABLE II

Precipitin Reactions of Cell Wall Lysates from Group N Streptococci, Strains C559 and B209

	Precipitin reactions with antiserum of stated group and type			
Cell wall lysates from	Group N		Group A*	Group D
	Type C559‡	Type B209‡	Type 61 (PGP antibody)	Type C3
Strain C559 (22°C culture)	++	+	+	++
Strain C559 (37°C culture)	++	—	—	++
Strain B209 (22°C culture)	+	++	+	—
Strain B209 (37°C culture)	—	++	—	—

+ Indicates positive reaction at cell wall concentration of 1 mg/ml.

++ Indicates positive reaction at cell wall concentration of 0.01 mg/ml.

* This antiserum contained antibody to unsubstituted Group A polyglycerophosphate (PGP).

‡ These antisera contained low-titered Group N antibody in addition to high-titered type-specific antibody.

it is probable that these reactions resulted from the presence of the group-specific polyglycerophosphate of Group N streptococci. In this regard it will be noted that the room temperature, but not the 37°C preparations from strains C559 and B209, reacted with a Group A polyglycerophosphate (PGP)[2] antiserum kindly provided by Dr. M. McCarty. The serological specificity of Group A PGP differs from that of Group N which contains galactose phosphate; Group A PGP does not precipitate in Group N antiserum but the reverse reaction does occur (4). There is evidence (4) that Group N streptococci contain a mixture of galactose-substituted and unsubstituted polyglycerophosphates (intracellular teichoic acids) and it was of interest to find that the latter, identified by precipitin tests, were rapidly liberated into the super-

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[2] *Abbreviation used in this paper:* PGP, polyglycerophosphate.

natant when B209 cocci grown at room temperature were resuspended in saline; this did not occur when the streptococci were grown at 37°C. One possible conclusion to be drawn from these findings is that the walls of these two strains of Group N streptococci were more permeable to PGP when grown at room temperature than they were after growth at 37°C.

Cross-reaction of strain C559 (Group N) with strain C3 (Group D): It will be seen from Table II that cell wall preparations from strain C559 cross-reacted with type-specific rabbit antiserum prepared against strain C3, a Group D streptococcus classified as *S. durans*. The reverse reaction also occurred; a cell wall preparation from strain C3 gave strong reactions with antiserum prepared against strain C559. The type and group antigens of strain C3

TABLE III

Inhibition of Precipitin Reactions between Strains C559 and B209 and Homologous Antisera by Various Sugars

Inhibitor 50 mg/ml	Inhibition	
	C559 reaction*	B209 reaction‡
	(%)	(%)
Galactose	4.6	29.5
Galactosamine	17.9	46.0
N-acetylgalactosamine	61.7	31.0
Glucose	6.1	54.4
Glucosamine	18.4	75.8
N-acetylglucosamine	39.8	83.5

* Strain C559 cell wall 30 μg + rabbit antiserum gamma globulin 98 μg in 1 ml of saline.
‡ Strain B209 cell wall 30 μg + rabbit antiserum gamma globulin 124 μg in 1 ml of saline.

have been the subject of a previous study (2) and it appears likely that the cross-reaction with strain C559 is due to a common cell wall carbohydrate. An immunological relationship between two different strains belonging to Group D and Group N has previously been described by Sharpe (1).

Specific inhibition of strain-specific precipitin reactions: In Group D, the type-specific serological reactivity of streptococcal cell wall carbohydrates is destroyed by 10 min of exposure to 100°C at pH 1 (2). The wall carbohydrates of the Group N cocci here investigated showed greater lability; with strain B209, reactivity was destroyed on heating to 100°C at pH 2 in 5 min, with strain C559, reactivity was lost on heating to 100°C for 5 min at a pH level between 1 and 2. In preliminary tests, the heat-inactivated cell wall material was found to be specifically inhibitory in precipitin reactions between the unheated carbohydrates and homologous antisera. Further investigation showed that incomplete inhibition could be achieved when known hexosamines were

substituted for the heat-inactivated cell wall material used in the preliminary tests. In concentrations of 50 mg/ml, N-acetylgalactosamine inhibited C559 reactions and N-acetylglucosamine the B209 reactions. The results of quantitative inhibition tests confirmed these findings and are set out in Table III.

These results show that the reaction of C559 cell walls with homologous antiserum is inhibited by both N-acetylgalactosamine and N-acetylglucosamine, the former being more inhibitory than the latter. In the nonacetylated form, these substances had little activity. With strain B209 and homologous antiserum, on the other hand, precipitation was strongly inhibited by N-acetylglucosamine but the nonacetylated hexosamine also had significant activity
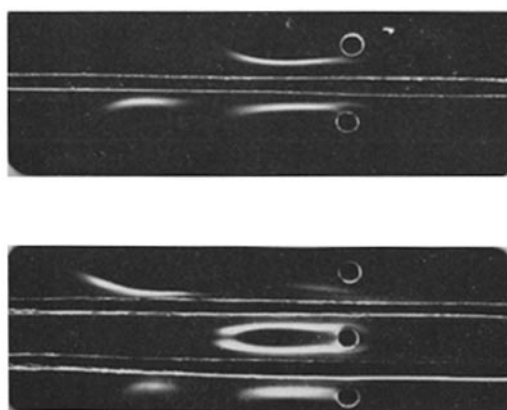

Fig. 1. Upper well contained C559 cell wall lysate, unheated; lower, heated to 100°C at pH 2; trough, type-specific antiserum (R1800). In Figs. 1–6 immunoelectrophoresis was in veronal buffer, pH 8.6, for 75 min at 5 ma.

Fig. 2. Upper well contained C559 extract made by heating intact cocci at pH 2 and 100°C; middle contained C559 cell wall lysate, unheated; lower contained C559 cell wall lysate heated to 100°C at pH 2; both troughs contained type-specific antiserum (R2014).

and some inhibition was produced by glucose itself. It is possible that the nonspecific inhibition shown in the reactions between strain B209 and its homologous antiserum was due, in part, to the high concentration in which the inhibitors were tested (50 mg/ml) and to the low titer of type-specific antibody in the available B209 antiserum (R1773).

The Composition and Structure of Cell Walls from Strain C559 (Orla Jensen).— Reference has already been made to the destructive effects of heat at low pH values upon the cell wall carbohydrates of Group N streptococci. While investigating this by means of immunoelectrophoresis, observations were made that provided further insight into the cell wall structure of strain C559.

When lysed cell walls of strain C559 were exposed for 2 min to a temperature of 100°C at pH 2, the cell wall material divided into two components

(Fractions A and B) revealed by immunoelectrophoresis at pH 8.6 using a cell wall-specific antiserum. Fraction A retained the mobility of the unheated material; Fraction B moved to the anode. This effect of heating was complete in 2 min and at pH 2 further heating of Fraction A, recovered by electrophoresis on Pevikon, produced no further change. These results are illustrated in Figs. 1 and 2.

The two components produced by heating cell wall material are clearly seen in the lower half of the slide depicted in Fig. 1. Before electrophoresis, an unheated cell wall lysate was placed in the upper well of the slide, and the same material after heating to 100°C at pH 2 in the lower well. The central
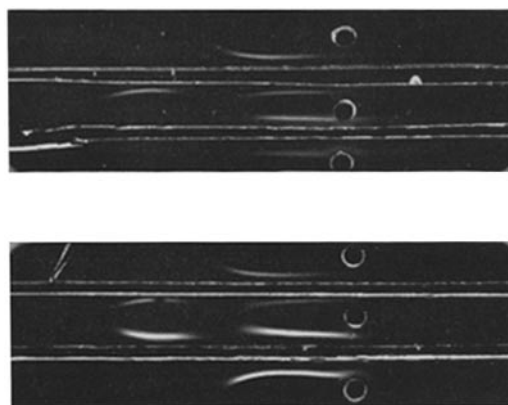

FIG. 3. Upper and lower wells contained C559 cell-wall lysate, unheated; middle well contained C559 cell-wall lysate heated to 100°C at pH 2; upper trough contained C3 (Group D) type-specific antiserum (R857); lower trough contained Group A-variant antiserum (R2842)

FIG. 4. Upper, middle, and lower wells contained the same as in Fig. 3. Upper trough contained C3 (Group D) antiserum (R857); Lower trough contained C559 (Group D) antiserum (R1800).

trough contained cell wall-specific antiserum (R1800). Fig. 2 compares an acid extract (10) of intact C559 streptococci (upper well) with a cell wall lysate, both heated (lower well) and unheated (middle well). The two troughs contained homologous antiserum (R2014). It can be seen that, like the heated cell wall lysate, the acid extract shows two components, but the proportion of Fraction B (mobile fraction) to Fraction A is greater in the acid extract than in the heated lysate.

These results showed that type-specific serological reactivity was shared by Fractions A and B. Further examination showed that both fractions also reacted with an antiserum prepared against the Group D strain, C3, already mentioned. The two fractions differed, however, in their reactivity with an antiserum prepared against a Group A-variant streptococcus, strain A486

(5); Fraction A reacted with this antiserum whereas Fraction B did not. The significance of this reactivity with Group A-variant antisera will be considered further at a later stage in this report.

Figs. 3–6 show the reactivity of Fractions A and B with an antiserum (R857) to Group D strain C3 and with an antiserum (R2842) to Group A-variant strain A486. Both fractions formed lines of precipitation with serum R857 which was placed in the upper troughs shown in Figs. 3 and 4. Fraction A formed a line with serum R2842 placed in the lower troughs, as shown in Figs. 3, 5, and 6; Fraction B failed to do so.
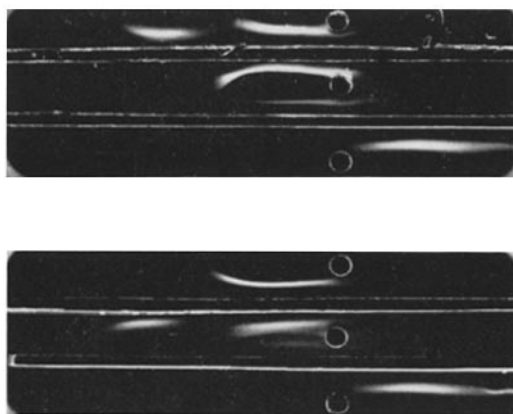

Fig. 5. Upper well contained C559 cell wall lysate heated to 100°C at pH 2; middle well contained C559 cell wall lysate unheated; lower well contained Group A-variant polysaccharide (1 mg/ml); upper trough contained C559 antiserum (R1800); lower trough contained Group A-variant antiserum (R2842).

Fig. 6. Upper and middle well contained the same as in Fig. 3. Lower well contained Group A-variant polysaccharide (1 mg/ml). The troughs contained the same as in Fig. 5.

The difference in serological reactivity of the two fractions made possible their isolation. This was achieved by electrophoresis of a heated cell wall lysate on Pevikon at pH 8.6. The fractions eluted from the Pevikon block were identified by their serological reactivity with homologous antiserum (R1800) and Group A-variant antiserum (R2842). By this means, each fraction was obtained in an amount sufficient for chemical analysis. For example, when a heated lysate from 30 mg of cell wall was subjected to electrophoresis, 19.01 mg of Fraction A and 2.04 mg of Fraction B were recovered by elution.

Tables IV and V present a comparison of the chemical composition and serological reactivity of Fractions A and B. In lysed cell walls the two fractions had an approximately 10:1 ratio by weight. The larger fraction (A) contained all of the cell wall mucopeptide and rhamnose together with part of the gluco-

samine and galactosamine. The smaller fraction (B) contained equal amounts of galactosamine and glucosamine, both in the N-acetylated form and probably linked as a polymer to an unidentified phosphorylated component. Type-specific serological activity was shared by the two fractions and was shown by inhibition tests to be associated particularly with the galactosamine component. Cross-reactivity with Group A-variant antiserum was found only in Fraction A, which contained almost all of the rhamnose. Cross-reactivity with Group D strain C3 antiserum was present in both fractions, but was not inhibited by galactosamine and the component responsible has yet to be identi-

TABLE IV

Chemical Composition of Two Fractions Isolated from Cell Walls from Group N Streptococci, Strain C559*

Cell wall component	Fraction A	Fraction B
	weight per 100 mg sample	weight per 100 mg sample
	(mg)	(mg)
Rhamnose	35.6	1.6
N-acetylglucosamine	17.1	12.6
N-acetylgalactosamine	12.3	12.6
Muramic acid phosphate	4.85	0
Lysine	2.7	0
Alanine	3.85	0
Glutamic acid	7.45	0
Aspartic acid	1.30	0
Phosphorus	0.78	2.1
Weight recovery‡	85.15	26.8

* See Table I for the analytical procedure.

‡ Weight recovery does not include moisture and ash. The phosphorus is included in the weight recovery only as a component of muramic acid phosphate.

fied. An earlier analysis of strain C3 cell walls showed that these contained rhamnose, hexosamine, glucose, and muramic acid (2).

The Immunological Relationship between Cell Wall Components of Group A-Variant and Group N Streptococci.—Since the specific reaction between strain B209 and homologous antiserum was inhibited by N-acetylglucosamine, and since N-acetylglucosamine is known to be the serological determinant of Group A cell wall polysaccharide (13, 14), it was obviously desirable to know whether an immunological relationship existed between the B209 and Group A polysaccharides. No reaction was observed between a number of potent Group A antisera and B209 cell wall carbohydrates, nor was any reaction detected between the latter and a rabbit antiserum to N-acetylglucosamine coupled with bovine serum albumin (14). To our surprise, however, strong precipita-

tion occurred when B209 cell wall carbohydrate was tested with Group A-variant antiserum.

It will be recalled that the cell wall carbohydrate of the Group A-variant streptococcus does not react, or does so only minimally, with Group A streptococcal antiserum in which the group antibody is directed toward the N-acetyl-glucosamine component of the cell wall antigen. Instead, the variant polysaccharide has a new specificity determined by the rhamnose component of the cell wall (13). Furthermore, the serological reactivity of the variant carbohydrate is destroyed by an enzyme produced by a soil bacillus; this enzyme has the ability to split, specifically, rhamnose oligosaccharides (13).

The variant antiserum used in the present investigation was provided by Dr. McCarty, and has been made by immunizing rabbits with a pepsin-digested Group A-variant streptococcus, strain A486. Confirmation that its reactivity with Group N strain B209 was attributable to a B209 cell wall carbohydrate with Group A-variant specificity came from the demonstration

TABLE V

Serological Reactivity of Fractions A and B Isolated from Cell Walls of Strain C559

Streptococcal antiserum prepared with	Precipitin reactions with cell wall fractions	
	Fraction A	Fraction B
Strain C559 (type-specific) (R1800)	+	+
Strain A486 (Group A-variant) (R2842)	+	—
Strain C3 (Group D) (R857)	+	+

that loss of reactivity with the variant antiserum occurred when the B209 carbohydrate was incubated with the variant-inactivating enzyme.

A similar cross-reactivity with Group A-variant antisera was shown by strain C559. Here, too, the cross-reaction was abolished by treatment of the cell wall lysate with variant-destroying enzyme. Immunoelectrophoretic patterns depicted in Figs. 3, 5, and 6 show that this cross-reactivity was confined to Fraction A of the C559 lysate; Fraction A contained virtually all of the cell wall rhamnose. It seems reasonable to conclude that the walls of strains C559 and B209 contained a Group A–variant-like antigen associated with the rhamnose component. Admittedly, this component in strain C559 had an electrophoretic mobility which differed from that of the variant carbohydrate (Figs. 5 and 6) but it must be remembered that Fraction A was a mixture of cell wall components (including muramic acid phosphate), whereas the variant carbohydrate had been partially purified.

The known relationship between Group N and Group D cell wall carbohydrates prompted us to see whether the latter also reacted with Group A-variant antisera. Of the four Group D strains examined (D76, C1, C3, and H69D5) all gave positive reactions when cell wall lysates (2) were tested, and

in all the reactivity was lost after treatment of the lysate with "variant enzyme". This enzyme had no effect on the reactions of the lysates with homologous cell wall-specific antisera.

In gel-diffusion precipitation reactions (Ouchterlony method), cell wall lysates from all of these Group N and Group D streptococci showed lines of identity with Group A-variant carbohydrates when tested with variant antiserum. This antiserum has been made with Group A strain 486, the source of the variant carbohydrate used in these experiments. Unfortunately, we did not have available for reciprocal tests Group D or Group N antisera that contained antibody to the variant carbohydrate.

Acid extracts (10) made from the Group N and Group D streptococci (excluding strain C3) had the same reactivity with Group A-variant antiserum

TABLE VI

Precipitin Reactions between Group A-Variant Antiserum and Normally Occurring Streptococci of Various Groups

Reactions of streptococcal extracts* with Group A-variant antiserum			
Positive reactions		Negative reactions	
Strep. group	No. of strains tested (all positive)	Strep. group	No. of strains tested (all negative)
D	4	B	5
E	4	F	5
G (small colony type)	3	G (large colony type)	3
M	3	H	5
N	2	L	3

In Groups A and C, rare mutants give positive reactions.

* Lysozyme digests of cell walls or acid extracts of intact cocci.

as had the corresponding lysed cell walls. In order to determine whether this relationship with the Group A-variant was shared by streptococci of other groups, extracts from these were tested against the Group A-variant antiserum before and after treatment with variant carbohydrate-destroying enzymes. Streptococci of Groups A and C were excluded from this survey since in these groups variant strains have already been identified.

The results of these tests are summarized in Table VI. It is perhaps significant that, with the exception of Group G, all members of each group investigated were consistent in their behavior with the variant antiserum, i.e., reactivity appeared to be a group characteristic. This was to be expected in Groups B, E, and F, and also in Group L[3] each of which is characterized by a specific cell wall carbohydrate, the "group antigen." All strains examined

[3] Elliott, S. D. Unpublished observation.

in Groups B, F, and L were negative; those in Group E were positive. Groups D and N, on the other hand, contain numerous different cell wall types, each characterized by a specific carbohydrate, so that it was surprising to find that all showed variant reactivity. Five Group H strains were negative and three Group M strains positive. Relevant information concerning the cell walls in Groups H and M is not available. Finally, in Group G we found three strains of each kind, reactive and nonreactive. Group G has a common cell wall carbohydrate and is divisible into two subgroups characterized by large and small colony forms (15). All three variant-reactive strains were of the small colony subgroup; the three negative reactors belonged to the large colony subgroup.

The positive reactions obtained with acid extracts of intact cocci were much weaker than those obtained with cell wall lysates. This was not surprising for it will be recalled that an acid extract of strain C559 examined by immuno-electrophoresis was found to contain mostly Fraction B and relatively little of Fraction A, the variant-reactive component (Fig. 2). The reverse was found in cell wall lysates; these contained Fractions A and B in a ratio of 10:1. From this it appears that heating at pH 2 releases from intact streptococci mainly cell wall-specific carbohydrate and relatively little of the underlying rhamnose component.

Clearly, this survey of strains from miscellaneous groups for Group A-variant reactivity was not intended to be an exhaustive search for unusual variants. Rather, it was for information concerning cross-reactivity between streptococcal cell wall polysaccharides and Group A-variant antiserum. The results showed that variant carbohydrate specificity is more widely distributed among streptococci than was previously thought to be the case.

DISCUSSION

The results here presented show that in the two strains of Group N streptococci investigated (C559 and B209) the cell wall polysaccharides are chemically different and serologically distinct. Our investigation into the structure of strain C559 was facilitated by the separation of a cell wall lysate into two serologically reactive fractions on heating at pH 2. We have been unable to repeat this procedure with strain B209 partly because there serological reactivity of the cell wall carbohydrate is destroyed by the conditions which in C559 lead to cleavage.

If our data concerning strain C559 are viewed in the light of existing knowledge concerning Group A streptococci, we can suggest a possible structure for the cell wall of the Group N microorganism; Strain C559 has, as the backbone of its wall structure, a mucopeptide layer to the outer surface of which there is probably attached a network of branching chains consisting predominantly of rhamnose. External to this is situated the serologically specific polymer of galactosamine and glucosamine. This may be attached to the terminal branches of the rhamnose network by the unidentified phosphorylated com-

ponent of the polymer. It would appear that in this connection there are two alternative linkages, one of which is labile at pH 2 and 100°C while the other is stable under these conditions. The latter would account for the galactosamine and part of the glucosamine that together with some type-specific serological reactivity remain in Fraction A after separation of Fraction B.

The remaining matter for discussion is more speculative and arises from our unexpected finding of an immunological relationship between the cell wall carbohydrate of variant Group A streptococci and wall carbohydrates from streptococci of other groups. McCarty showed that variant specificity of Group A cell walls resides in the rhamnose moiety of the carbohydrate (13). For the purposes of this discussion we shall refer to the Group A variant-reactive component in streptococci of heterologous groups as the "variant-like antigen." Previous workers have shown that this occurs in certain Group C strains (6, 7). Our investigations show that it is present also in the walls of streptococci belonging to Groups D, E, G, M, and N. In these groups the variant-like antigen appears to coexist with cell wall-specific carbohydrates. These streptococci, therefore, resemble Group A and Group C intermediate strains that react not only with variant but also with Group A or Group C antisera (5, 6). We failed to demonstrate the presence of the variant-like antigen in streptococci of Groups B, F, H, and L and in three of six strains in Group G. In assessing these negative results, it is important to remember that overtly variant strains are rarely encountered in Groups A and C. On the other hand, the variant-like antigen can be shown to be an invariable component of normal Group A streptococci where it can be unmasked by enzymatic degradation of the cell walls. From lack of the appropriate degrading enzyme, the variant-like antigen has not been sought in normal Group C streptococci but, from analogy with Group A, it seems not unlikely that it is there. Although we have no direct supporting evidence, we would go further and suggest that possibly a variant-like antigen exists, either masked or overt, in all streptococci whose walls contain rhamnose. Without the specific enzymes required for degradation of the walls, this contention is hard to prove but the problem is under active investigation.

With the information here presented we can begin to trace in the walls of streptococci a structure resembling that found in the walls of Salmonella (16). The variant-like antigen of the streptococcus appears to be the counterpart of the rough antigen of Salmonella. Each is attached to a rigid cell wall backbone formed in streptococci by the mucopeptide component. Each gives attachment to immunologically specific cell wall polysaccharides by which its own serological reactivity may be masked. Each, in the absence of the specific polysaccharides, assumes a dominant role in the serological reactivity of the cell wall and is the source of cross-reactions between strains that normally show a high degree of specificity.

SUMMARY

A Group A variant-like antigen has been detected in streptococci belonging to Groups D, E, G, M, and N. In Groups D and N the variant-like antigen was located in the streptococcal cell walls.

In two strains of Group N streptococci (C559 and B209) the cell walls were chemically different and serologically distinct. In strain C559 N-acetylgalactosamine, and in strain B209, N-acetylglucosamine were the major determinants of serological specificity.

The cell walls of strain C559 contained at least three serologically reactive components: a rhamnose-containing fraction that precipitated with an antiserum to Group A-variant carbohydrate; a strain-specific polysaccharide composed of galactosamine and glucosamine, both in the N-acetylated form and probably polymerized with an unidentified phosphorylated substance; and a component of unknown composition serologically related to a Group D streptococcus strain C3 ($S.$ $durans$). An analogy is drawn between the cell wall structure in streptococcus and $Salmonella$.

BIBLIOGRAPHY

1. Sharpe, M. E. 1952. The occurrence of a common type antigen in streptococci of Groups D and N. *J. Gen. Microbiol.* **7**:192.
2. Elliott, S. D. 1960. Type and group polysaccharides of Group D streptococci. *J. Exp. Med.* **111**:621.
3. Elliott, S. D. 1962. Teichoic acid and the group antigen of Group D streptococci. *Nature (London).* **193**:1105.
4. Elliott, S. D. 1963. Teichoic acid and the group antigen of lactic streptococci. *Nature (London).* **200**:1184.
5. McCarty, M., and R. C. Lancefield. 1955. Variation in the group-specific carbohydrate of Group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains. *J. Exp. Med.* **102**:11.
6. Krause, R. M., and M. McCarty. 1962. Variation in the group-specific carbohydrate of Group C hemolytic streptococci. *J. Exp. Med.* **116**:131.
7. Araujo, P., and R. M. Krause. 1963. Group-specific carbohydrates of Group C variant hemolytic streptococci. *J. Exp. Med.* **118**:1059.
8. Swift, H. F., A. T. Wilson, and R. C. Lancefield. 1943. Typing Group A hemolytic streptococci by M. precipitin reactions in capillary pipettes. *J. Exp. Med.* **78**:127.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
10. Lancefield, R. C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. *J. Exp. Med.* **57**:571.

11. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methyl pentoses and a spectrophotometric micro method for their determination. *J. Biol. Chem.* **175**:595.
12. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756.
13. McCarty, M. 1956. Variation in the group-specific carbohydrate of Group A streptococci. II. Studies on the chemical basis for serological specificity carbohydrates. *J. Exp. Med.* **104**:629.
14. McCarty, M. 1958. Further studies on the chemical basis for serological specificity of Group A streptococcal carbohydrate. *J. Exp. Med.* **108**:311.
15. Lancefield, R. C. 1940–41. Specific relationship of cell composition to biological activity of hemolytic streptococci. *Harvey Lect.* **36**:251.
16. Luderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of Salmonella and related enterobacteriaceae. *Bacteriol. Rev.*