THE CELL WALLS OF GROUP D STREPTOCOCCI*

I. The Immunochemistry of the Type 1 Carbohydrate

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The type-specific carbohydrates of Group D streptococci are components of the cell wall and are the structural and chemical counterparts of the specific carbohydrates of Groups A, B, C, and G streptococci (1). Although a number of serologic types of Group D streptococci have been identified, the immunochemical basis for this antigenic classification has not been elucidated (2). This report describes the isolation and properties of the type antigens of Group D Type 1 (*Streptococcus faecalis* var. *zymogenes*) and Type 26 (*Streptococcus faecium* var. *durans*).

The Group D antigen, unlike the group-specific carbohydrates for most of the other streptococcal groups, is a cytoplasmic constituent and not a cell wall component (1, 3), and has been identified as a glucose-glucosyl glycerol teichoic acid (4). Isolation and purification of the type antigen is simplified by the fact that Group D cell walls are devoid of the group antigen.

Methods and Materials

Streptococcal Strains.—Group D cell walls were prepared from strains D76 (Type 1) and C3 (Type 26) obtained from Dr. Rebecca C. Lancefield, The Rockefeller Institute.

Preparation of Cell Walls.—Cell walls were prepared using the Braun homogenizer as described previously (5).

Muralytic Enzymes.—Egg white lysozyme was obtained from Mann Research Labs, New York. The *Streptomyces albus* enzyme was prepared as previously described (6).

Preparation of Cell Wall Carbohydrates.—Carbohydrate was extracted from D76 cell walls by several techniques.

Formamide extraction: The hot formamide method of Fuller (7), as modified by Krause and McCarty (8), was used to extract carbohydrate which was further purified by alcohol and acetone precipitation and with resin treatment (9).

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Streptomyces albus enzyme extraction: Cell walls, previously heated for 30 minutes at 60°C to destroy autolytic activity, were suspended in 0.07 M phosphate buffer (pH 8) and incubated at 37°C with the *S. albus* enzyme for 20 hours. Chloroform was used as a preservative. The resulting lysate was deproteinized by the method of McCarty (10), and the water-soluble carbohydrate purified as in the formamide procedure.

Egg white lysozyme extraction: Cell walls, previously heated to 60°C for 30 minutes to destroy autolysin, were suspended in 0.07 M phosphate buffer (pH 6.2) containing lysozyme at 125 μg per ml. Lysis was allowed to proceed in a dialysis sack for 20 hours at 37°C against 10 volumes of the buffer. Merthiolate at a final concentration of 1:10,000 was employed as a preservative. Upon completion of lysis, the lysate was clarified by centrifugation (25,000 *g* for 30 minutes), followed by deproteinization as described above, and the carbohydrate was recovered and purified as in the formamide procedure.

Analytical Methods.—Quantitative determinations of rhamnose, hexosamines (measured as glucosamine), muramic acid, and amino acids were performed by methods described previously (8). Glucose and galactose were measured on hydrolyzed material using the "glucostat" and "galactostat" reagents, respectively (Worthington Biochemical Corporation, Freehold, New Jersey).

Precipitin Analysis.—Quantitative precipitin analyses were performed according to the method of McCarty and Lancefield (11). Antisera against Group D and Type 1 antigens were generously supplied by Dr. R. C. Lancefield. Other rabbit antisera were prepared by methods previously described (11).

EXPERIMENTAL

Formamide Extraction of Type 1 Carbohydrate.—Previous reports have identified a carbohydrate and the mucopeptide as the two major constituents of

TABLE I

Composition of Type 1 (D76) Cell Walls, Formamide Extracted Carbohydrate, and Residue

	Cell walls	Formamide treatment	
		Carbohydrate	Residue
	per cent	*per cent*	*per cent*
Rhamnose	18.7	37.6	< 1.0
Hexosamine*	10.9	11.5	10.2
Glucose	12.9	19.8	< 1.0
Galactose	‡	‡	‡
Muramic acid	3.8	< 1.0	9.6
Alanine	9.7	< 1.0	23.5
Glutamic acid	4.2	< 1.0	10.6
Lysine	5.1	< 1.0	11.6

* Although both glucosamine and galactosamine are present, total content for both sugars was calculated on the basis of the glucosamine standard. This convention is employed for the remainder of the tables.

‡ Galactose, although not measured quantitatively, was detectable in trace amounts by paper chromatography.

trypsinized cell walls of hemolytic streptococci, including those of Group D (1, 8). Presented in Table I are the chemical compositions of Group D Type 1 cell walls, the carbohydrate extracted with hot formamide, and the mucopeptide residue. As is the case for the other groups of streptococci so far examined, the bulk of the cell wall rhamnose is recovered in a soluble carbohydrate, but the mucopeptide residue is essentially devoid of this sugar. The other sugars in the carbohydrate, in addition to rhamnose, are hexosamines (glucosamine and galactosamine), glucose, and galactose. The galactose content was not deter-

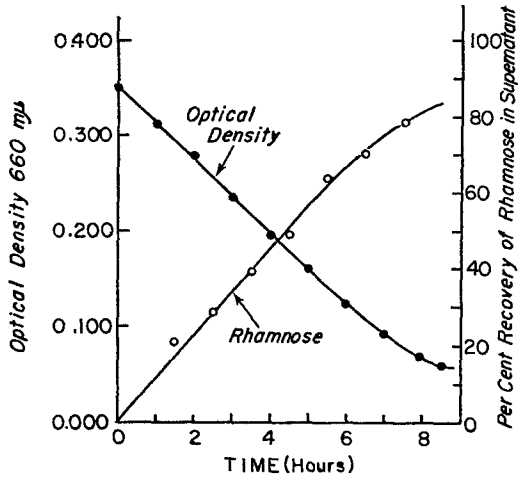

FIG. 1. Autolysis of D76 cell walls in 0.07 M phosphate buffer pH 6.2 at 37°C. The reaction was measured by the decrease in optical density of the cell wall suspension and by the detection of rhamnose in the supernatant following centrifugation of aliquots of the suspension.

mined because galactosamine interfered with the "galactostat" procedure, however, a paper chromatogram of a carbohydrate hydrolysate yields a spot representative of galactose which is much less prominent than the spots representing the other sugars. Thus, it is probable that the percentage of galactose is minimal.

It should be emphasized that the cell wall carbohydrate, extracted by the hot formamide procedure, is completely devoid of immunologic reactivity.

Extraction of Carbohydrate by Autolysis, and the S. Albus Enzyme and Lysozyme Procedures.—Because the hot formamide procedure yields serologically inactive carbohydrate, attention was directed to other methods for the extraction of the type antigen from the cell walls. Procedures which have proved effective in the past for lysis of the cell wall and the release of the antigen employ the *S. albus* enzyme preparation and crystalline egg white lysozyme (1). In addition to the use of these two enzymatic procedures, a useful third alternative

was discovered during the course of this work. Under properly controlled conditions Group D Type 1 cell walls (strain D76) undergo spontaneous autolysis with release within 6 to 8 hours of 80 per cent of the cell wall rhamnose as a component of the soluble type antigen.

For autolysis experiments, cell walls were prepared from a washed suspension of Type 1 streptococci, strain D76, by disruption in a Braun homogenizer operated at 2000 RPM for 10 minutes. Although disruption is more rapid if the machine is run at 4000 RPM, the resulting cell walls have little or not autolytic activity. The cell walls were separated from the other cellular elements by differential centrifugation and washed repeatedly with distilled water. Nucleic acids and proteins are removed by treatment with nucleases and trypsin. Autolysis does not occur prior to treatment with trypsin. Because autolysis of the walls proceeds maximally at pH 6.0–6.2 and is markedly diminished at pH 7.5–7.8, the enzymatic treatment was carried out at the higher pH. The cell walls, after repeated washing with distilled water in the cold, were stored in the lyophilized state.

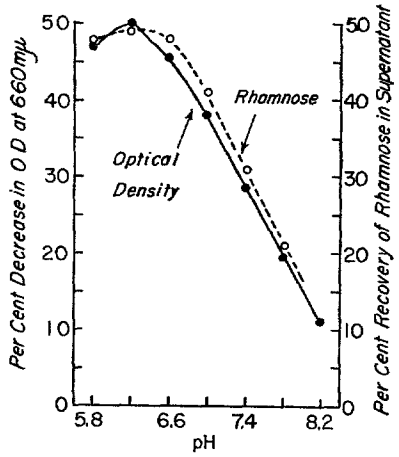

FIG. 2. Determination of pH optimum of the autolytic activity of D76 cell walls. The cell walls were suspended in 0.07 M phosphate buffer at different pH values, incubated at 37°C, and autolysis terminated at 4.5 hours. The degree of autolysis was measured by calculating the per cent decrease in the optical density, and by detecting the per cent of the initial rhamnose in the supernatant following centrifugation of the test suspension.

The autolysis of the cell walls, suspended in 0.07 M phosphate buffer pH 6.2 at 37°C is recorded in Fig. 1. Lysis of the cell walls was detected by the decrease in the optical density of the suspension and by the measurement of the rhamnose in the soluble material. To measure rhamnose, autolysis was terminated in an aliquot by heating at 60°C for 10 minutes. The aliquot was centrifuged at 4°C at 25,000 *g* for 30 minutes, and the rhamnose content of the supernatant determined. The rhamnose in the supernatant was plotted as per cent recovery of the total initial cell wall rhamnose in the aliquot. It is to be noted that autolysis continued until the bulk of the cell walls is solubilized.

The optimal pH for autolysis was determined by the experiment depicted in Fig. 2. In this experiment the cell walls were suspended in phosphate buffers covering a pH range from 5.8 to 8.2. Autolysis was terminated after 4.5 hours, and the extent of autolysis was determined as in the previous experiment. In this instance the change in turbidity during this time period is plotted as per cent decrease in OD at 660 mμ.

Autolysis of bulk lots of cell walls for the subsequent isolation of the type carbohydrate was carried out as follows: five hundred mg of trypsinized cell walls were suspended in 0.07 M phosphate buffer pH 6.2 and incubated at 37°C for 12 hours. Chloroform was used as a preservative. The carbohydrate was purified from the lysate by the acid-alcohol and acetone precipitation procedure

TABLE II

Composition of Type 1 (D76) Carbohydrates Extracted by Four Different Methods

	Extraction procedure			
	Hot formamide	Autolysis	*S. albus* enzyme	Lysozyme
	per cent	*per cent*	*per cent*	*per cent*
Rhamnose	37.6	24.6	30.5	22.5
Hexosamine	11.5	15.7	14.9	11.9
Glucose	19.8	15.6	16.3	14.4
Muramic acid	<1.0	3.4	2.8	4.2
Alanine	<1.0	5.3	0.9	11.7
Glutamic acid	<1.0	1.6	0.3	5.5
Lysine	<1.0	2.4	0.6	5.8

followed by resin treatment (9). The same procedures were employed to purify the carbohydrate from the lysate as were employed in the formamide extraction method.

The data in Table II afford a comparison between the chemical composition of the formamide carbohydrate and that obtained by autolysis, and the *S. albus* enzyme, and lysozyme procedures. It is to be noted that the formamide carbohydrate is devoid of mucopeptide while the carbohydrates obtained with the enzymes contain an appreciable amount of this material. The low content of the amino acids in the *S. albus* carbohydrate is probably due to the fact that the *S. albus* enzyme solution contains an amidase or a peptidase. Purified lysozyme does not have such activity and this is reflected in the high content of amino acids in the lysozyme carbohydrate. The autolytic carbohydrate has a higher content of amino acids than that in the *S. albus* carbohydrate, but a lower content than that in cell walls. This diminution of the amino acid content may be dependent upon amidase or proteolytic activity during the autolysis process. All of the carbohydrates, except the formamide carbohydrate, are re-

active with homologous type-specific antisera, but are unreactive with Group D antisera. The higher content of rhamnose, hexosamine, and glucose, and the lower content of the mucopeptide components in the case of the *S. albus* carbohydrate suggests that a greater purification of antigen is achieved with the *S. albus* enzyme than with lysozyme or the autolysis procedure.

Split Products Released from Cell Walls by Enzymatic Action.—Previous studies indicate that cell wall dissolving enzymes release dialyzable and non-dialyzable soluble mucopeptide in addition to the carbohydrate (12). Similar experiments were undertaken to identify the soluble substances of Type 1 cell walls after autolysis and lysis with lysozyme.

TABLE III

*Dialyzable and Non-Dialyzable Soluble Fractions of Type 1 (D76) Cell Walls Lysed by Autolysis**

	Non-dialyzable fractions			Dialyzable fraction
	Acid-alcohol precipitated fraction	Acetone-precipitated fraction	Acetone-soluble fraction	
	μmole	*μmole*	*μmole*	*μmole*
Rhamnose	8	516	0	0
Glucose	3	297	0	0
Hexosamine	2	302	4	6
Muramic acid	1	47	5	9

* The mucopeptide amino acids, alanine, lysine, and glutamic acid, were detected qualitatively by paper chromatography in each fraction.

Autolysis: Five hundred mg of trypsinized cell walls were allowed to autolyze in 100 ml of 0.07 M phosphate buffer pH 6.2 for 12 hours at 37°C. The lysate was then dialyzed against two changes of large volumes of water for 36 hours at 4°C. The combined dialysis fluid was evaporated to 50 ml and labeled dialyzable fraction. The dialyzed autolysate was centrifuged at 25,000 *g* for 30 minutes to separate the fine particulate residue from the soluble materials. The supernatant was treated with 2 volumes of acid-alcohol, and the resulting precipitate suspended in 10 ml water and labeled acid-alcohol precipitated fraction. To this supernatant was added 5 volumes of acetone. The resulting precipitate was collected and labeled acetone-precipitated fraction, and the supernatant was evaporated to 10 ml and labeled acetone-soluble fraction.

Lysis with lysozyme: Five hundred mg of heat-inactivated trypsinized cell walls were suspended in 100 ml of 0.07 M phosphate buffer pH 6.2 and were lysed with lysozyme at a final concentration of 125 μg per ml. Digestion continued for 20 hours at 37°C while dialyzing against 10 volumes of buffer. The dialysis fluid was subsequently evaporated to 50 ml. The lysate was then treated with acid-alcohol and acetone, and fractions prepared and labeled as above.

The results of the autolysis experiment are presented in Table III. Non-dialyzable acetone-soluble material and dialyzable material were devoid of rhamnose. The detection of material containing muramic acid in the acetone-soluble

fraction, as well as in the dialyzable fraction, is indicative of the heterogeneous size of the soluble mucopeptide polymers. It should be emphasized, however, that these fractions comprise less than 25 per cent of the total mucopeptide in the cell walls, because the bulk of this substance was recovered with the acetone-precipitated carbohydrate.

The results of the lysozyme experiment are tabulated in Table IV. At least one-half of the rhamnose is recovered in the initial alcohol precipitation, and most of the remainder was recovered in the subsequent acetone precipitation. Less than 25 per cent of the total muramic acid was recovered in the dialyzable and the acetone-soluble fractions. This suggests that only a small portion of the total mucopeptide, devoid of carbohydrate, is liberated by lysozyme.

TABLE IV

Dialyzable and Non-Dialyzable Soluble Fractions of Type 1 (D76) Cell Walls Lysed with Lysozyme

	Non-dialyzable fractions			Dialyzable Fraction
	Acid-alcohol precipitated fraction	Acetone-precipitated fraction	Acetone-soluble fraction	
	μmole	*μmole*	*μmole*	*μmole*
Rhamnose	204	197	<1	<1
Glucose	138	120	4	3
Hexosamine	114	99	4	3
Muramic acid	18	25	8	5

The major difference between the split products obtained with lysozyme and those obtained with autolysis merits comment. Approximately one-half of the serologically-reactive carbohydrate was precipitated from the cell wall lysozyme digest by alcohol, and the other half by the subsequent addition of acetone to the alcoholic solution. On the other hand, only minimal carbohydrate was precipitated from the cell wall autolysate by alcohol, whereas the bulk was precipitated after the addition of acetone.

Acid Hydrolysis of the Type 1 Carbohydrate.—Sharpe has reported that the serologic reactivity of the type carbohydrates of certain strains of Group D streptococci is destroyed by treatment at 100°C at pH 1.5 (2). This observation, confirmed in the present studies, suggests that the antigenic determinant is an acid-labile component of the carbohydrate. Experiments were devised to identify the labile fraction and to determine if it inhibits the serologic reaction between the intact antigen and homologous antibody.

A solution of autolytic carbohydrate, adjusted to pH 1.5 with HCl, was heated at 100°C for 20 minutes. After neutralization the solution was dialyzed against cold water. The dialyzing fluid was concentrated and labeled dialyzable fraction. Five volumes of acetone were added to the non-dialyzable material and the precipitate collected by centrifugation, dissolved in water,

and labeled acetone-precipitated fraction. The supernatant was saved and labeled non-dialyzable acetone-soluble fraction.

Analyses of the fractions of carbohydrate obtained after hydrolysis at pH 1.5 are tabulated in Table V. Approximately 9 per cent of the glucose and 11 per cent of the hexosamine, initially present in the carbohydrate, were recovered in the dialyzable and acetone-soluble fractions, whereas all except a minimal amount of the total rhamnose was recovered in the acetone-precipitated fraction. These findings suggest that rhamnose is not a feature of the acid-labile component of the type carbohydrate, but that hexosamine and glucose may be part of the immunologically reactive portion of the antigen. This view was substantiated by the fact that the dialyzable material was a potent inhibitor of the

TABLE V

Dialyzable and Non-Dialyzable Soluble Fractions of Type 1 (D76) Carbohydrate after Hydrolysis at 100°C at pH 1.5

	Non-Dialyzable fractions		Dialyzable fraction
	Acetone-precipitated fraction	Acetone-soluble fraction	
	μmole	*μmole*	*μmole*
Rhamnose	329	4	2
Glucose	176	6	10
Hexosamine	183	15	9
Muramic acid	29	<1	<1

precipitin reaction between Type 1 antigen and homologous antibody. For instance, the dialyzable material, at a final concentration in terms of glucose content of 0.3 micromoles per ml, causes 80 per cent inhibition of the quantitative precipitin reaction when tested at antigen-antibody equivalence.

Precipitin Inhibition with Mono- and Disaccharides.—Quantitative precipitin inhibition studies were undertaken to ascertain whether glucose or one of the hexosamines was a significant component of the antigenic determinant of the Type 1 carbohydrate. The quantitative precipitin inhibition tests were carried out at antigen-antibody equivalence. *S. albus* carbohydrate was employed as the antigen. Final concentration of antigen was 30 μg per ml and the final concentration of sugar inhibitor was 55 μmole per ml. In addition to the known constituent sugars of the carbohydrate, a number of other sugars were also tested.

From an inspection of the data in Table VI it is clear that D-glucose and *N*-acetylglucosamine are effective inhibitors. In addition, D-fucose, D-galactose, and L-arabinose, which have the same symmetry about carbons 2 and 3 as that of D-glucose, also inhibit the reaction. If glucose is altered at the third carbon by methylation, the product is not inhibitory, however, 2-deoxy-D-glucose, and

N-acetylglucosamine, which represent alterations at the second carbon, have inhibitory capacity similar to D-glucose.

The inhibitions with disaccharides are presented in Table VII. Sucrose and maltose, both alpha glucosides, are particularly effective inhibitors of the pre-

TABLE VI

Inhibition of Type 1 (D76) Precipitin Reaction by Monosaccharides

Inhibitor	Inhibition
55 μmole/ml	*per cent*
N-Acetylglucosamine	59.4
N-Acetylgalactosamine	16.1
D-Glucose	59.0
L-Glucose	19.0
D-Galactose	45.1
D-Fucose	51.2
L-Fucose	22.9
L-Arabinose	42.0
D-Arabinose	12.3
L-Rhamnose	0.0
D-Mannose	24.7
L-Mannose	3.1
2-Deoxy-D-Glucose	43.1
3-O-Methyl-D-Glucose	9.8

All precipitin reactions were carried out at antigen-antibody equivalence.

TABLE VII

Inhibition of Type 1 (D76) Precipitin Reaction by Disaccharides and Methylglucosides

Inhibitor	Inhibition
55 μmole/ml	*per cent*
Sucrose	100.0
Maltose	96.7
D-Cellobiose	18.2
β-Lactose	13.6
α-Methyl-D-Glucose	100.0
β-Methyl-D-Glucose	12.2

All precipitin reactions were carried out at antigen-antibody equivalence.

cipitin reaction, whereas the beta glucoside, D-cellobiose, identical to maltose except for the beta linkage between the glucosyl residues, is an ineffective inhibitor. Results of other precipitin inhibition studies with maltose indicate that 45 per cent inhibition could be achieved with as little as 14 μmole per ml. In addition, α-methyl-D-glucose was inhibitory, whereas β-methyl substituted glucose was not inhibitory.

These studies suggest that both glucose and *N*-acetylglucosamine may be major components of the determinant and that the terminal residue has an alpha linkage to the remainder of the carbohydrate. Current studies are in progress to identify the terminal sugar of the determinant.

Composition of Type 26 Cell Walls.—The composition of Type 26 cell walls is similar in many respects to that of Type 1. The same sugars and amino acids are present, although Type 26 also contains minimal amounts of aspartic acid and glycine which are not found in the cell walls of Type 1. Presented in Table VIII are the chemical compositions of the cell walls, the type carbohydrate

TABLE VIII

Composition of Type 26 (C3) Cell Walls, Formamide Extracted Carbohydrate and Residue, and Lysozyme Carbohydrate

	Cell walls	Formamide extraction		Lysozyme carbohydrate
		Carbohydrate	Residue	
	per cent	*per cent*	*per cent*	*per cent*
Rhamnose	18.4	22.8	1.0	23.2
Hexosamine*	17.1	22.5	11.7	16.9
Glucose	6.0	11.5	<1.0	7.0
Galactose	‡	‡	‡	‡
Muramic acid	4.8	<1.0	10.0	4.5
Alanine	7.4	<1.0	14.0	4.7
Glutamic acid	6.2	<1.0	12.9	3.9
Lysine	4.2	<1.0	8.3	3.8
Aspartic acid	1.7	<1.0	2.8	1.0
Glycine	<1.0	<1.0	1.0	<1.0

* Although both glucosamine and galactosamine are present, the total content for both sugars was calculated on the basis of the glucosamine standard.

‡ Galactose, although not measured quantitatively, was detectable in trace amounts by paper chromatography.

extracted with hot formamide, and the mucopeptide residue. The chemical components of the carbohydrates are similar to those of Type 1. It should be stressed that Type 26 carbohydrate, extracted with formamide, is serologically reactive whereas the Type 1 carbohydrate is not.

The type carbohydrate can also be extracted from the Type 26 cell wall with *S. albus* enzyme and lysozyme. The composition of the lysozyme carbohydrate is also presented in Table VIII. This carbohydrate has a high content of mucopeptide, and on a weight basis the formamide carbohydrate is serologically more reactive than the lysozyme carbohydrate.

Despite the similarity between the chemical composition of Type 26 carbohydrate and that of Type 1, the two antigens are serologically distinct. Neither carbohydrate reacts with the opposite antiserum. Furthermore, although glucose and *N*-acetylglucosamine are effective inhibitors of the Type 1 precipitin

reaction, these and the other constituent sugars of Type 26 carbohydrate do not cause appreciable inhibition of the Type 26 reaction.

DISCUSSION

Attention has been directed in this report to the chemical nature of the type antigens of Group D streptococci. These carbohydrates, the chemical and structural analogs for the group antigens for most other hemolytic streptococci, contain an appreciable quantity of rhamnose. In the case of Groups A and C carbohydrates, the serologic reactivity of the rhamnose disaccharide is masked by specific terminal *N*-acetylhexosaminide residues (13), and in the case of Groups B and G carbohydrates rhamnose monosaccharide is a major feature of the antigenic determinants (14). On the basis of the work presented here there is no evidence that rhamnose occupies a similar critical position in the antigenic structure of Types 1 and 26 carbohydrates.

The haptenic inhibition studies indicate that both D-glucose and *N*-acetylglucosamine may be components of the antigenic determinant of Type 1 carbohydrate. Inhibition with maltose but not with cellobiose suggests that the terminal residue has an alpha linkage to the subterminal sugar. This view is corroborated by inhibition studies with α- and β-methyl glucosides. Furthermore, the structural configuration about the third carbon atom of the determinant sugar seems to be an important feature of the hapten. Each inhibiting monosaccharide possesses an identical configuration at carbon three, and methylation of this atom produces a non-inhibiting derivative.

The precipitin reaction between Type 26 antigen and antibody is not inhibited to any appreciable extent by either the constituent sugars or the disaccharides. Thus, this carbohydrate has little antigenic similarity to the Type 1 carbohydrate.

During the course of this work autolysis of Type 1 cell walls was employed as an effective means to extract the carbohydrate. Certain features of this autolytic process merit emphasis. Autolysis is initiated after exposure of cell walls to a proteolytic enzyme, and only a small percentage of the mucopeptide, devoid of the carbohydrate moiety, is released as soluble material. This is in contrast to the action of muralytic enzymes on the cell walls of Groups A and C streptococci in which a major portion of the mucopeptide is dialyzable after hydrolysis (12). Young *et al.* have studied the autolysin of *Bacillus subtilis* cell walls in considerable detail (15). In this case autolysis is not dependent upon proteolytic activation. Although there is a release of only minimal quantities of dialyzable mucopeptide after autolysis, when cell walls are treated with lysozyme, the bulk of the mucopeptide is dialyzable. Because of these and other data, it is suggested that the lytic action of *B. subtilis* autolysin is dependent upon an amidase, whereas the primary action of lysozyme is hydrolysis of glycosidic bonds in the mucopeptide (15, 16). Studies are in progress to clarify the chemical nature of the autolytic process in the case of Group D Type 1 strep-

tococci. It should be noted, however, that both lysozyme and autolysis liberate only a small fraction of the total cell wall mucopeptides as free dialyzable material. Thus, on the basis of the work reported here, autolysis and lysozyme may have a similar lytic effect on Group D Type 1 cell walls.

SUMMARY

Group D Types 1 and 26 cell walls and the corresponding type-specific carbohydrates, extracted from the walls by various means, contain rhamnose, glucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. Mucopeptide elements are also present in the walls and in enzymatically-extracted carbohydrates. Types 1 and 26 carbohydrates extracted by formamide contain no mucopeptide, but the serologic reactivity of Type 1 antigen is destroyed by this procedure. The Type 1 antigen was successfully extracted, however, by a new technique involving autolysis of cell walls at pH 6.2. The type carbohydrate prepared by this procedure has chemical and serological similarities to the antigens prepared by the *S. albus* enzyme and the lysozyme methods.

Quantitative precipitin inhibition studies with Type 1 antigen and antibody indicate that D-glucose and *N*-acetylglucosamine may be components of the antigenic determinant. The terminal residue is probably bound by an β-glycosidic bond to the subterminal sugar.

Similar studies with the Type 26 carbohydrate have not revealed any of the chemical features of the antigenic determinant.

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