STUDIES ON THE CHEMICAL STRUCTURE OF THE STREPTOCOCCAL CELL WALL*

I. The Identification of a Mucopeptide in the Cell Walls of Groups A and A-Variant Streptococci

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Plate 19

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Recent studies on the chemical composition of the bacterial cell wall indicate that the basic structural component, which is responsible for the rigidity of the wall, is similar in all bacteria examined (1). This component, designated as the cell wall mucopeptide, is composed of *N*-acetylglucosamine, *N*-acetylmuramic acid (3-O-lactyl-*N*-acetylglucosamine), and a limited number of amino acids. In the case of streptococci these two amino sugars and alanine, lysine, glycine, and glutamic acid have been identified in hydrolysates of trypsinized cell walls. However, this analysis of hydrolysates does not provide information on the arrangement of these substances in the chemical structure of the cell wall. The data reported here indicate that the two amino sugars and the four amino acids do in fact comprise a mucopeptide fraction. This substance is distinct from the other major cell wall component, the group-specific carbohydrate hapten, which contains rhamnose and *N*-acetylglucosamine.

The work of Salton (2) has shown that the lysis of certain bacteria by lysozyme is accompanied by the release of the cell wall mucopeptide into soluble fragments. Lysis of streptococcal cell wall by phage-associated lysin is shown to be associated with a similar release of fragments of mucopeptide. These findings support the growing evidence which suggests that the specific linkage split by lysozyme and other cell wall–dissolving enzymes resides in the mucopeptide fraction.

Materials and Methods

Streptococcal Strains.—Group A streptococcal strains T12, D58, and S43/100, and Group A-variant strains T27A and K43 var. were used to prepare cell walls.

Preparation of Cell Walls.—The cell walls were prepared by the method of Salton and Horne which employs a Mickle disintegrator (3). Differential centrifugation was used to separate the cell walls from the fine particulate material; and the walls were then treated with trypsin in phosphate-buffered saline, dialyzed against water, and finally lyophilized.

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Muralytic Enzymes.—Muralytic enzyme has been used by Murphy as a generic term for those enzymes which lyse bacteria by dissolving the cell wall (4). In this investigation three such enzymes were used.

The lysozyme was a crystalline preparation obtained from Worthington Biochemical Sales Company, Freehold, New Jersey.

The *Streptomyces albus* enzyme was prepared as previously described (5).

The phage-associated lysin was prepared as previously described (6) but with the following modifications. The phage lysate was brought to 0.6 saturation with ammonium sulfate in the cold and stored for 3 to 7 days at 4°C. The fine precipitate was collected by centrifugation at 10,000 RPM in the Spinco centrifuge with the 21S head. The precipitate was taken up in phosphate-buffered saline, pH 7, at $\frac{1}{50}$ the original lysate volume, the insoluble residue discarded, and the remaining solution brought to 0.3 saturation with ammonium sulfate. The precipitate obtained was discarded by centrifugation at 15,000 RPM with the 30S head, and the supernatant brought to 0.5 saturation with ammonium sulfate. The precipitate that formed at this point was collected by centrifugation at 15,000 RPM with the 30S head, taken up in a small volume of phosphate-buffered saline, and the insoluble residue removed from the enzyme solution by centrifugation. The enzyme solution was dialyzed against large volumes of saline phosphate buffer pH 7.2 for 18 hours. After removal of the phage by ultracentrifugation as previously described, the enzyme preparation was stored frozen in the dry ice box.

Preparation of the Cell Wall Carbohydrate.—Group-specific carbohydrate was prepared from cell walls with the phage-associated lysin and the *Streptomyces albus* enzymes by previously described methods (6).

Analytical Methods.—Rhamnose was determined by the method of Dische and Shettles (7). The hexosamines were estimated as glucosamine by a modification of the Elson and Morgan procedure (8). Determinations of glucosamine and muramic acid were made after they had been separated by the method of J. T. Park as described by Perkins and Rogers (9). The glucosamine standard was recrystallized glucosamine hydrochloride. Standardization of muramic acid determinations was carried out with a preparation of uridine diphospho–muramic acid–peptide generously supplied by J. T. Park. The muramic acid content of this preparation was established by estimation of *N*-acetylmuramic acid after hydrolysis in 0.1 N HCl for 4 minutes, using the method of Reissig *et al.* for analysis (10). When used as a standard for free muramic acid, the UDP-muramic-peptide was hydrolyzed under the same conditions employed with the cell wall fractions for hexosamine analysis: *i.e.*, 4 hours in 2 N HCl.

In accordance with the results of Crumpton (11), the Elson-Morgan procedure was modified by making readings at both 505 and 530 mμ immediately and after 18 hours at room temperature. Glucosamine values were estimated on the basis of the immediate 530 mμ readings, and muramic acid values on the 18 hour 505 mμ readings. In addition, the ratio of the optical density at 505 and 530 mμ after 18 hours provided evidence for characterization of the hexosamines as suggested by Crumpton (11). This 505/530 ratio ranged from 0.8 to 0.9 for glucosamine and 2.5 to 3.0 for muramic acid. Mixtures of the two substances gave intermediate ratios which provided an index of the relative amounts present.

Identification of Amino Acids.—The material for analysis was hydrolyzed and quantitative determination of the amino acids was carried out by the paper chromatographic method of Mandelstam and Rogers (12). In general, 60 to 70 hours rather than the 48 hours described provided a better separation of the amino acids.

Formamide Extraction.—Extraction of cell walls with formamide was carried out by Fuller's procedure (13). Both lyophilized material and cell walls packed by centrifugation were employed with comparable results. Some variability in extractability was encountered, but in most instances the formamide suspension was heated with stirring at 170°C in an oil bath for 20 minutes. The insoluble residue could be recovered by direct centrifugation of the

extract, but the separation was facilitated by precipitating with 2 to 2.5 volumes of acid alcohol according to Fuller. This resulted in precipitation of the residue free from significant amounts of the soluble fraction. The soluble carbohydrate was recovered from the alcohol supernate by the addition of acetone and could then be purified by the same procedures employed with material obtained by enzymatic lysis of cell walls.

EXPERIMENTAL

Previous investigations have identified rhamnose, glucosamine, muramic acid, and alanine, lysine, glutamic acid, and glycine as constituents of the streptococcal cell wall (14) and as will be shown here the same constituents are found in the group-specific carbohydrate hapten isolated from it by muralytic

TABLE I

Chemical Composition of Streptococcal Cell Walls, and the Group-Specific Carbohydrate

	Group A cell walls strain T12	Group A-variant cell walls strain T27A	Enzyme Group A carbohydrate	Acid Group A carbohydrate
	per cent	*per cent*	*per cent*	*per cent*
Rhamnose	34.0	35.0	40.0	63.2
Glucosamine	18.8	5.9	22.0	26.0
Muramic acid	6.4	5.9	1.6	—
Alanine	17.4	20.4	5.9	—
Glutamic acid	7.9	9.9	2.4	—
Lysine	8.0	7.9	2.4	—
Glycine	1.0	1.2	0.2	—

enzymes. Data on the percentage composition of such a carbohydrate isolated by phage muralytic enzyme are included in Table I. The bulk of the rhamnose initially present in the cell wall prior to digestion with enzyme was subsequently recovered in the purified carbohydrate. This is not the case, however, with the amino sugars and the amino acids. Approximately one-fourth of the initial cell wall muramic acid, for instance, was recovered in the group carbohydrate obtained with lytic enzymes. Such a result suggested that the cell wall contained a secondary structure composed of muramic acid and amino acids in addition to the group-specific hapten. Experiments were devised, therefore, to identify substances other than the group-specific carbohydrate which had been solubilized by the phage muralytic enzyme.

Trypsinized cell walls were prepared from strain T12 streptococci, and dissolved with phage-associated lysin. A heavy turbid suspension, after 8 hours of digestion at 37°C, became opalescent. The material was dialyzed in the cold against four changes of distilled water; the dialysates were combined and concentrated *in vacuo* to a small volume. The dialyzed digestion mixture was centrifuged at 4000 RPM in the angle-head centrifuge to separate the fine particulate residue from the soluble cell wall material. This residue, a dark grey substance, was

washed by centrifugation several times with water and the washings added to the original non-dialyzable soluble cell wall material. In order to isolate the serologically active group-specific carbohydrate from this latter material, the protein was first precipitated by adding 2 volumes of absolute alcohol and removed by centrifugation. The carbohydrate was precipitated from the alcohol-water supernatant by the addition of 4 volumes of acetone and collected by centrifugation. The acetone-alcohol-water mixture, containing any non-dialyzable material remaining after removal of the above protein and carbohydrate, was concentrated *in vacuo* to a small volume. Chemical and serological analyses were performed on five fractions, labeled as follows: the insoluble cell wall residue, the non-dialyzable alcohol-precipitable fraction, the non-dialyzable acetone-precipitable fraction, the non-dialyzable acetone-soluble fraction, and the dialyzable material.

TABLE II

Dialyzable and Non-Dialyzable Soluble Fractions of T12 Cell Walls Lysed with Phage-Associated Lysin

	Non-dialyzable fraction		Dialyzable fraction
	Acetone-precipitated fraction	Acetone-soluble fraction	
	μmols	*μmols*	*μmols*
Rhamnose	1445	2	5
Glucosamine	671	42	105
Muramic acid	53	41	88
Alanine	548	260	*
Glutamic acid	167	55	*
Lysine	183	91	*
Glycine	63	55	*

* Because the dialyzable fraction contains the electrolytes of the digestion mixture, quantitative determination of the amino acids was performed after dialyzable material had been eluted from a darco G60 column as recorded in Table III.

Nearly complete lysis of the cell walls by the phage-associated lysin is indicated by the fact that the insoluble residue represents only a small fraction of the initial weight of the cell wall and contains less than 0.1 per cent of the rhamnose and glucosamine. Rhamnose, glucosamine, muramic acid, and amino acid values after acid hydrolysis of various non-dialyzable and dialyzable cell wall fractions are presented in Table II. Because the non-dialyzable alcohol-precipitated fraction was essentially devoid of rhamnose and amino sugars it is not included in the table. As would be expected, almost all of the rhamnose was found in the non-dialyzable acetone-precipitated fraction, and serological testing with Group A antiserum indicated that this was the primary fraction with group-specific serologic activity. The muramic acid, glucosamine, and amino acids were distributed among the three fractions represented in Table II, although quantitative data for amino acids on the dialyzable material are not given because it contained electrolytes which interfered with the amino acid

assay. This information was obtained, as will be indicated in the next section, after fractions of the dialyzable material had been eluted from a darco G60 column. Both the non-dialyzable acetone-soluble fraction and the dialyzable material are composed of the elements of mucopeptide. However, an additional portion of the muramic acid and amino acids is associated with the acetone-precipitable fraction which contains the group-specific carbohydrate.

Additional experiments were undertaken to determine the nature of the dialyzable material. Analysis prior to hydrolysis with the Elson-Morgan reaction revealed only traces of hexosamine, while after hydrolysis, both glucosamine and muramic acid were readily detected indicating that the dialysate contained an oligosaccharide of amino sugars. As with the case of the amino sugars, the

TABLE III

Comparison of the Mole Ratios of Cell Wall, and the Soluble Cell Wall Products following Lysis by Phage-Associated Enzyme

	Group A cell wall	Non-dialyzable mucopeptide	Dialyzable mucopeptide eluted from a darco G60 column	
			25 per cent alcohol eluate	50 per cent alcohol eluate
Muramic acid	(1)	(1)	(1)	(1)
Glucosamine	4.1	1.0	1.2	1.2
Alanine	11.0	6.3	8.2	7.3
Glutamic acid	3.6	1.3	2.3	2.2
Lysine	3.4	2.2	2.8	1.9
Glycine	1.1	1.3	0.7	1.1

amino acids were separable on paper chromatography only after hydrolysis. The following experiment, in which the dialyzable material was eluted from a darco G60 column with various alcohol-water mixtures, indicated that the amino sugars and the peptide were combined into a single unit.

An aliquot of the dialyzable fraction of the previous experiment, in which T12 streptococcal cell walls were lysed with phage muralytic enzyme, was loaded onto a darco G60–celite 503 column. Equal parts of darco G60 and celite 503 were added to a 1.9 cm diameter column in a water slurry, and the final column length after settling was 17.5 cm. The hydrolyzed dialysate loaded onto the column contained 12 mg of hexosamine. The column was first eluted with 500 ml of H_2O, and then successively with 2 500 ml volumes of 15 per cent alcohol, 25 per cent alcohol, and 50 per cent alcohol. The eluates were concentrated *in vacuo* to approximately 10 ml and then analyzed for the amino sugars and amino acids.

The chemical analysis of the eluates from the column are included in Table III. Data for the second 25 and 50 per cent eluates are omitted because the analysis indicated that all detectable material collected from the column with a particular alcohol-water mixture was removed in the first eluate. No

material was removed from the column by water or 15 per cent alcohol. The first 25 and 50 per cent eluates, on the other hand, contained nearly equal amounts of both amino acids and amino sugars, and the composition as determined by calculating the mole ratios were similar. The amounts of glucosamine and muramic acid approached a 1:1 mole ratio, and that of alanine and lysine 3:1. The fact that no detectable material was eluted from the column by a concentration of alcohol below 25 per cent indicated that the amino sugars and the amino acids were structurally associated. The occurrence of separate fractions from the charcoal column is interpreted as evidence that mucopeptide fractions of different sizes are present in the dialyzable material.

Table III also allows a comparison of the chemical composition of the dialyzable material as eluted from the darco G60 column and the non-dialyzable mucopeptide (the acetone-soluble material of Table II) with that of trypsinized Group A cell walls. With the exception of glucosamine, the relative amounts of the several constituents are of the same order of magnitude in the intact cell wall as in the products obtained by enzymatic lysis. The group-specific carbohydrate is responsible for the high glucosamine value in the cell wall. The identification of split products which contain glucosamine, muramic acid, alanine, lysine, glycine, and glutamic acid, following lysis of the cell wall with phage muralytic enzyme supports the view that a cell wall mucopeptide is the substrate for the enzymic action. Other methods of hydrolysis were employed next to investigate the mucopeptide component of the wall.

Acid Hydrolysis.—The initial work on the serological classification of hemolytic streptococci was dependent on the isolation of a serologically active group-specific substance by treatment with boiling at pH 2 (15). This substance, purified from the extraction mixture, is a carbohydrate with the chemical composition shown in Table I. This table compares the composition of carbohydrate preparations extracted with acid and with phage muralytic enzyme. Both substances are equally active serologically, as indicated in quantitative precipitin tests. Both acid- and enzyme-extracted carbohydrates contain rhamnose, but the acid-extracted material does not contain muramic acid or the amino acids.

An examination of the cell wall residue following a single period of acid hydrolysis reveals that only a part of the serologically active carbohydrate has been removed. Even repeated acid extractions leave an appreciable quantity of serologically active carbohydrate in the residue. At the same time, however, detectable amounts of mucopeptide are not removed from the cell wall residue by this repeated treatment with acid. Thus, acid hydrolysis has been useful to separate the serologically active carbohydrate from the residual mucopeptide, but it has not been possible to obtain a mucopeptide residue which does not contain an appreciable quantity of group-specific carbohydrate. This goal is more nearly achieved by the use of formamide extraction.

Formamide Extraction.—The use of hot formamide to extract polysaccharides from bacteria was introduced by Fuller (13). A heavy suspension of bacteria becomes almost clear in the course of this treatment, and it was originally assumed that the organisms were completely dissolved with extensive destruction of many components but preservation of polysaccharides. However, closer examination of the products reveals that a formamide-insoluble residue is consistently present. When this method of extraction is applied to isolated streptococcal cell walls rather than to intact bacteria, it is apparent that the insoluble residue represents an appreciable fraction of the total material. Clear evidence for the separation of the mucopeptide and the serologically active carbohydrate was obtained by analysis of the soluble and insoluble fractions.

In a typical experiment, trypsinized cell walls of strain T12 were extracted with formamide by the procedure outlined under Methods. The soluble fraction, representing the serologically active carbohydrate, contained 95 per cent of the total rhamnose recovered. Complete removal of rhamnose from the insoluble residue was not achieved even by repeated extraction, but preparations were consistently obtained in which rhamnose comprised less than 5 per cent of the dry weight of the residue. Thus, the removal of serologically active carbohydrate is almost complete.

Despite the vigorous nature of the formamide treatment, it was found that the residue occurred in the form of particles which retain the characteristic disc-like appearance of the original cell walls when examined by phase microscopy. This is better illustrated by electron microscopic examination, and the electron micrograph presented in Fig. 1 demonstrates that the formamide residue is composed of structures comparable in appearance to untreated cell walls. A comparison of these electron micrographs with those of cell walls suggests that the formamide residue structures are less dense.

The results of rhamnose and hexosamine analyses of formamide residues prepared from different strains of Group A and Group A-variant streptococci are presented in Table IV in comparison with the analyses of the original cell walls and of the soluble carbohydrate obtained from the formamide extract. As noted above, the residues contain less than 5 per cent rhamnose in each case while the original cell walls contain 21 to 36 per cent. Glucosamine and muramic acid are present in the residues in approximately equal amounts. In contrast to this finding, the soluble carbohydrates contain large amounts of rhamnose and no detectable muramic acid. In addition, comparison of the rhamnose and glucosamine values between the Group A and A-variant carbohydrates reveals a striking contrast in composition. The Group A carbohydrate contains approximately 60 per cent rhamnose and 30 per cent glucosamine, while the Group A-variant has over 80 per cent rhamnose and only small amounts of glucosamine. These values are in the same direction as observed previously for the two

carbohydrates prepared by enzymatic lysis of cell walls and are consistent with published findings on the chemical basis for the serological differentiation of the carbohydrates. It is evident that the formamide carbohydrates differ from the enzyme preparations chiefly by the elimination of elements of the mucopeptide.

It would appear that the formamide residues represent the mucopeptide portion of the cell wall. This is supported by the data presented in Table V which includes the hexosamine and amino acid analyses of formamide residues

TABLE IV

Composition of Group A and Group A-Variant Cell Walls; and the Soluble Carbohydrate and the Insoluble Residue following Formamide Extraction

		Strains of streptococci			
		Group A		Group A-variant	
		D58	T12	T27A	K43 var.
		per cent	*per cent*	*per cent*	*per cent*
Cell walls	Rhamnose	21.0	34.0	35.0	36.0
	Glucosamine	10.4	18.8	5.9	7.2
	Muramic acid	3.6	6.4	5.9	5.0
Formamide-extracted carbohydrate	Rhamnose	60.0	58.8	85.0	80.5
	Glucosamine	30.0	25.0	3.0	1.4
	Muramic acid	—	—	—	—
Formamide residue	Rhamnose	2.4	4.9	4.0	1.2
	Glucosamine	10.0	11.0	6.9	6.1
	Muramic acid	9.3	9.4	6.8	10.7

of Group A and A-variant strains of streptococci. The similarity of the figures for the three strains examined suggests that a common mucopeptide structure is present in both of these streptococcal groups. The mole fractions of alanine, glutamic acid, and lysine approach the ratio 3:1:1 which has been found in the mucopeptide of other bacteria. Only small amounts of glycine were detected in addition to variable traces of other amino acids. The two amino sugars and four amino acids account for 65 to 70 per cent of the total weight of the residue. Because the lyophilized residue contains approximately 10 per cent water and the amino sugars are *N*-acetylated in the native material, the total percentage accounted for is higher than indicated by the values in Table V.

It should be pointed out that complete recovery of the total rhamnose and amino sugars of the cell wall is not achieved after formamide treatment. This loss, which may be as high as 30 per cent, probably reflects some destruction of the sugars during exposure to formamide at high temperatures. However,

there is no evidence for alteration of the two major fractions, as indicated (*a*) by the serological activity of the soluble carbohydrate which is quantitatively equivalent to that prepared with enzyme and (*b*) the susceptibility of the mucopeptide residue to muralytic enzymes, discussed in the next section.

Lysis of the Formamide Residue.—The demonstration that the streptococcal cell wall contains a rigid structure composed essentially of mucopeptide and that the phage muralytic enzyme releases dialyzable glucosamine–muramic acid–peptide units during lysis of the cell wall, suggests that as with other organisms the substrate for the various muralytic enzymes is the mucopeptide. Of the three muralytic enzymes considered here, *Streptomyces albus* enzyme,

TABLE V

Composition of Residue after Extraction of Cell Walls with Formamide

	Streptococcal strain					
	Group A				Group A-variant T27A	
	T12		D58			
	per cent	*mole ratio*	*per cent*	*mole ratio*	*per cent*	*mole ratio*
Muramic acid	9.4	(1)	9.3	(1)	6.8	(1)
Glucosamine	11.0	1.5	10.0	1.5	6.9	1.4
Alanine	31.0	8.7	26.6	8.1	22.7	9.4
Glutamic acid	17.0	2.9	14.0	2.6	12.6	3.2
Lysine	14.0	2.4	14.6	2.6	11.2	2.9
Glycine	0.9	0.3	0.8	0.3	0.7	0.3

Rhamnose values, omitted from this table, are included in Table IV.

phage-associated lysin, and lysozyme, only the latter does not lyse the whole organism or the isolated cell wall. It was of particular interest to find, therefore, that all three enzymes lysed the formamide mucopeptide residue.

10 mg samples of D58 formamide residue, and 5 mg samples of T12 formamide residue were incubated with each one of the muralytic enzymes, lysozyme, *S. albus* enzyme, and phage-associated lysin. Lysozyme was present in a final concentration of 0.5 mg/ml. No absolute standard is available to estimate the amount of the *S. albus* enzyme or the phage lysin. Sufficient enzyme was present, however, to lyse cell walls in a much greater concentration than that of the formamide residue used here. A final sample of each formamide residue was treated with phosphate buffer and served as a control. After the addition of the enzymes the tubes were incubated at 37°C for 18 hours. They were centrifuged in the cold at 10,000 RPM for 1 hour and the supernatant poured off and saved for subsequent analysis. The residues were washed several times with water, suspended in a known volume of water, and also analyzed along with the supernatants for total hexosamine.

Table VI gives the percentage distribution of the recovered hexosamine between the enzymic extracts and the insoluble residues. It is clear that all of the mucopeptide substrate was solubilized by the *S. albus* enzyme, and while

dissolution of the substrate by the other two enzymes was incomplete, more than one-half of the initial hexosamine was in solution after lysis. The unexpected finding that lysozyme dissolved the streptococcal mucopeptide, in view of the fact that it does not attack the whole cell wall, fortifies the belief that the formamide residue is a structural mucopeptide which is the substrate for muralytic enzymes. These data suggest that the whole cell wall is not sensitive to lysozyme until after the removal with formamide of the group carbohydrate. Such treatment exposes the mucopeptide so that it is sensitive to lysozyme. The *S. albus* and phage muralytic enzymes readily attack the mucopeptide in the intact cell wall, and do not require the removal of the group carbohydrate.

TABLE VI

Lysis of Formamide Residue with Muralytic Enzymes

Formamide residue	Fraction	Per cent of total hexosamine recovered after treatment with enzymes			
		Lysozyme	Phage lysin	*S. albus* enzyme	Saline control
		per cent	*per cent*	*per cent*	*per cent*
D58	Extract	72	58	100	0
	Residue	28	42	0	100
T12	Extract	68	80	98	2
	Residue	32	20	2	98

DISCUSSION

In previous studies *Streptomyces albus* enzymes (5) and phage-associated lysin (6) have been employed to dissolve the streptococcal cell wall. The group-specific polysaccharide was isolated from the lysates and attention was directed primarily to the serological characteristics, chemical composition, and the chemical basis for serological specificity of this substance. In the experiments reported here these enzymes, as well as various other measures, have been employed to hydrolyze the cell wall; and an examination of the substances released by these procedures has suggested that the cell wall is composed of two carbohydrate substances: the characteristic group-specific polysaccharide and a separate entity designated as the cell wall mucopeptide.

The mucopeptide contains glucosamine, muramic acid, and a limited number of amino acids; and a similar substance has been identified in the cell wall of many bacteria (1). Muramic acid was first detected in spore peptides by Strange and Powell (16), and the proposed structure, confirmed by synthesis by Strange and Kent, is 3-O-lactyl-D-glucosamine (17). A consistent feature of mucopeptide isolated from a wide range of bacteria is that the proportion of glucos-

amine to muramic acid approaches a mole ratio of 1:1. The amino acid composition may vary somewhat but frequently alanine, lysine, and glutamic acid are present in a ratio of 3:1:1. Ghuysen and Salton (18) have shown that during lysis of *Micrococcus lysodeikticus* cell walls with lysozyme, several dialyzable glucosamine–muramic acid–peptide products and a non-dialyzable fraction are released. The dialyzable products include a disaccharide of N-acetylmuramic acid and *N*-acetylglucosamine, a complex of these two sugars, and several peptide–acetyl amino sugar complexes. It seems clear that these substances comprise a mucopeptide in the intact cell wall, and that lysis of the cell wall by lysozyme occurs because the polymer is hydrolyzed. The occurrence of mucopeptide in a wide variety of different bacteria implies that it is a key component of the bacterial cell, and there is now considerable evidence to support the suggestion that it is the structural substance of the cell wall.

In the work reported here the cell walls of Groups A and A-variant streptococci have been lysed with phage-associated lysin and a dialyzable glucosamine–muramic acid–peptide complex identified in addition to the nondialyzable group-specific polysaccharide. In this dialyzable complex the glucosamine:muramic acid mole ratio approached 1:1, and the alanine:lysine:glutamic acid ratio approached 3:1:1. These findings suggested that the intact cell wall was composed of a mucopeptide polymer and the group-specific polysaccharide.

The separate status of the polysaccharide and the mucopeptide is more clearly demonstrated by the formamide extraction procedure. The two fractions obtained when cell walls are extracted with formamide are a soluble nondialyzable group-specific polysaccharide and an insoluble glucosamine–muramic acid–peptide residue. The group-specific polysaccharide extracted by this technique is serologically identical with that extracted with *S. albus* enzyme or the phage-associated lysin. There is considerable difference, however, in the chemical composition. More than 90 per cent of the formamide polysaccharide and but only two-thirds of the enzyme polysaccharide is accounted for by rhamnose and *N*-acetylglucosamine. This difference is due to the fact that the latter substance also contains muramic acid and peptide.

Previous work has stressed the underlying chemical similarity of Groups A and A-variant polysaccharides obtained with *S. albus* enzymes (19). The special feature which distinguishes these two serologically, however, is that the Group A polysaccharide contains side chains of *N*-acetylglucosamine. The serological and chemical properties of the underlying rhamnose structure for both polysaccharides are similar. The polysaccharides employed in this previous work contained mucopeptide, because they were obtained by enzymatic lysis of the cell wall. It has been difficult, therefore, to assess the role of glucosamine in a central structure of the polysaccharides so prepared. The composition of the formamide polysaccharides, on the other hand, throws some light upon this

question. It is particularly interesting to note the composition of the formamide Group A–variant polysaccharide. This substance contains 80 to 85 per cent rhamnose and only 1 to 3 per cent glucosamine, and it therefore seems unlikely that glucosamine has a key position in the central structure of the variant polysaccharide molecule. In the case of Group A streptococci, the formamide polysaccharide contains 60 per cent rhamnose and 25 per cent glucosamine. If a comparison can be made to the Group A-variant formamide polysaccharide, and the previous work suggests this is reasonable, then the Group A formamide polysaccharide also contains a rhamnose central structure which is essentially devoid of glucosamine. The high percentage of glucosamine in the Group A material represents in large part the terminal β-*N*-acetylglucosaminide residues which determine serological specificity.

The other product obtained by formamide extraction of the cell walls is an insoluble residue composed of particles with a discrete disc shape similar to the cell wall. Chemical analysis revealed glucosamine and muramic acid in a mole ratio of 1:0.7, and alanine, glutamic acid, and lysine in a ratio of 3:1:1. This composition resembles that of the dialyzable amino sugar–amino acid complex released from the cell wall by phage-associated lysin. The evidence suggests that the mucopeptide structure isolated by formamide represents the substrate in the intact cell wall for the muralytic enzymes. *S. albus* enzyme, phage-associated lysin, and lysozyme release a soluble glucosamine–muramic acid–peptide complex from the formamide residue. The degree of homogeneity of the soluble substance is not known, nor has it been determined whether the substances released by the three enzymes are identical. The lysis of formamide residue by lysozyme is of particular interest, because this enzyme does not act on the intact streptococcal cell wall.[1] This suggests that the removal of the group polysaccharide from the cell wall by formamide has exposed the linkages of the mucopeptide residue which are hydrolyzed by lysozyme.

It seems clear that different linkages in the whole cell wall have been hydrolyzed by formamide on the one hand and the enzymes on the other. The enzymes release soluble amino sugar–peptide complexes and therefore probably act on a glycosidic linkage in the mucopeptide polymer. The release of the serologically reactive polysaccharide moiety by formamide suggests that hydrolysis has occurred between this fraction and the mucopeptide.

SUMMARY

Lysis of trypsinized Group A streptococcal cell walls with phage-associated lysin releases into solution dialyzable and non-dialyzable mucopeptide fractions composed of *N*-acetylglucosamine, *N*-acetylmuramic acid and alanine, glutamic

[1] Prior to the experiments reported here, preliminary tests suggested that the mucopeptide fraction obtained by formamide extraction was not susceptible to the lytic action of lysozyme. This preliminary view was expressed in discussion at a recent symposium (20).

acid, lysine, and glycine in addition to the characteristic group-specific carbohydrate. The latter substance contains appreciable amounts of *N*-acetylmuramic acid and the amino acids as well as *N*-acetylglucosamine and rhamnose.

Hot formamide extraction of the cell walls results in a soluble fraction of group-specific carbohydrate and an insoluble residue. The Group A carbohydrate in this instance is composed of rhamnose and *N*-acetylglucosamine. The composition of the insoluble residue is similar to that of the mucopeptide fractions released from the cell wall by phage-associated lysin. This residue was shown by electron microscopy to be composed of discrete discs which appear similar in structure to the intact cell wall.

The specific carbohydrate obtained by hot formamide extraction of Group A–variant cell walls was composed almost exclusively of rhamnose. The residue fraction was similar to that of Group A.

The residue of cell walls extracted with hot formamide is extensively solubilized not only by phage-associated lysin and *S. albus* enzyme, but also by lysozyme, which has no measurable effect on the intact streptococcal cell wall.

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EXPLANATION OF PLATE

PLATE 19

FIG. 1. Electron micrograph of hot formamide extraction residue of T12 cell walls. These discrete disc structures are composed of mucopeptide. The preparation has been shadowed with chromium. Magnification approximately × 15,000.

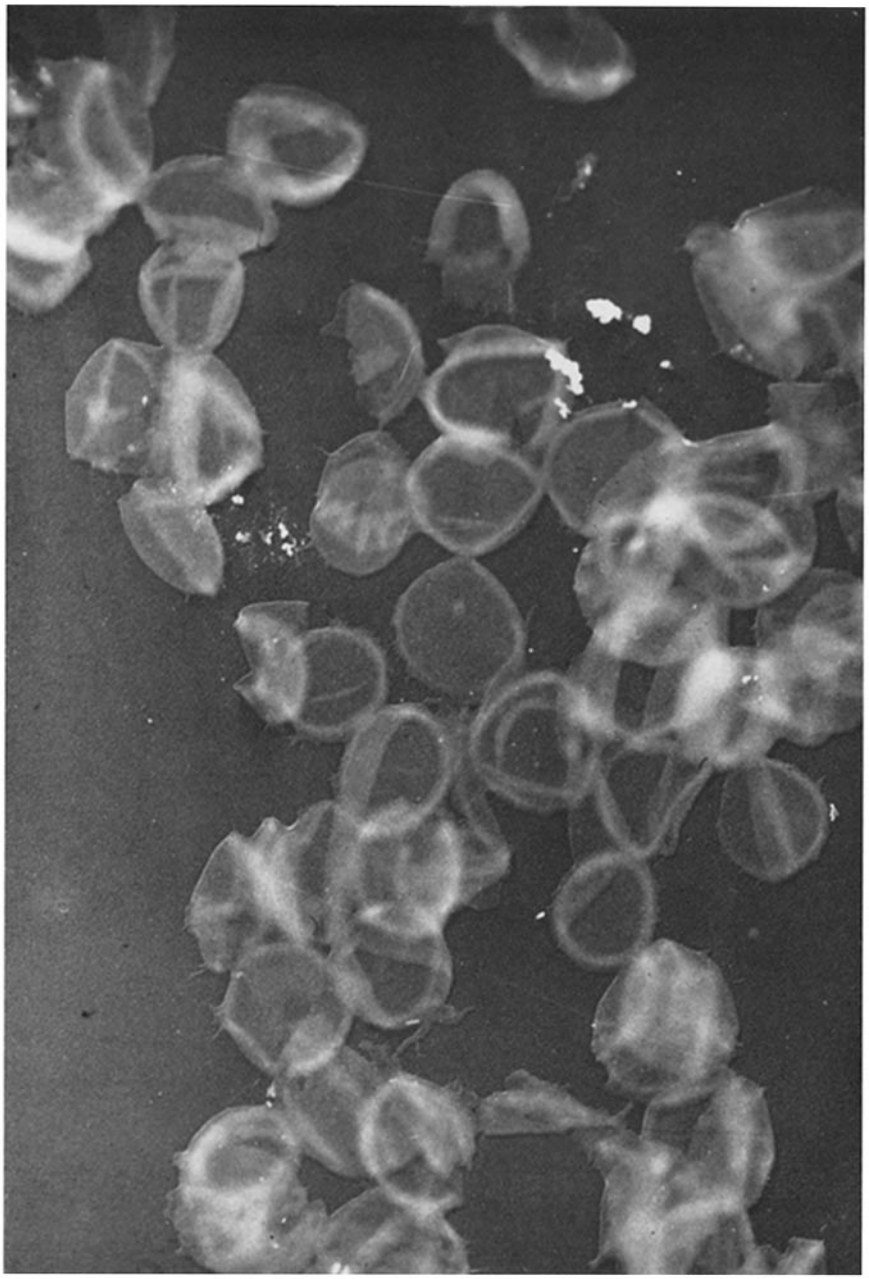

FIG. 1

(Krause and McCarty: Cell wall mucopeptide of streptococci)