STUDIES OF STREPTOCOCCAL CELL WALLS

III. The Amino Acids of the Trypsin-Treated Cell Wall[1]

JAMES A. HAYASHI and S. S. BARKULIS

Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

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Previous papers have described the preparation of cell walls of group A hemolytic streptococci (Barkulis and Jones, 1957) and studies on their antigenicity in rabbits (Barkulis *et al.*, 1958). The present paper describes the results of a quantitative study of the amino acids of trypsin-treated cell walls of group A streptococci. In addition, the results of similar studies of the C polysaccharide of cell walls are described. The polysaccharide constitutes 50 to 60 per cent of the dry weight of trypsin-treated cell walls and, when obtained in a soluble form by enzymatic hydrolysis (McCarty, 1952*b*), contains a small amount of amino acid. A comparison of the amino acid content of the trypsin-treated cell wall and of the C polysaccharide suggests that glutamic acid, lysine, and alanine occur in defined, recurring units in the cell wall and are attached to a rhamnose-hexosamine polymer.

The presence of large amounts of lysine, glutamic acid, alanine, and glucosamine in acid hydrolyzates of streptococcal cell walls has been shown by Salton (1953) and by Cummins and Harris (1956) using paper chromatographic methods. Their chromatograms also showed the presence of a ninhydrin-reactive substance which the latter authors believed was identical with a new amino sugar found in the spores of *Bacillus megaterium* by Strange and Powell (1954). This new amino sugar, called muramic acid, has been associated primarily with the bacterial cell wall (Work, 1957) or with the postulated intermediates of cell wall synthesis, the uridine nucleotides which accumulate in cultures of penicillin-inhibited *Staphylococcus aureus* (Park and Strominger, 1957). Amino acid analyses of the cell walls of the latter organisms reported by Park and Strominger (1957) indicate the presence of muramic acid, alanine, lysine, and glutamic acid. Cifonelli and Dorfman (1957) stated that they found muramic acid in the streptococcal cell wall, but did not present details of their work. Strange (1956) has advanced a formulation for muramic acid as 3-O-carboxyethylglucosamine, and Kent (1957) has synthesized DL-3-O-carboxyethylglucosamine and reported that it is similar to authentic muramic acid.

The results of the analyses reported here are consistent with the work cited and allow a basis for postulating a tentative structure for the streptococcal cell wall.

METHODS

Preparation of cell walls and polysaccharide. Cell walls were prepared as described by Barkulis and Jones (1957) using type 14, group A hemolytic streptococci. In the later stages of this work, a Nossal disintegrator (Nossal, 1953) was used for breaking cells. This instrument proved more convenient since heavier suspensions of cells would be disintegrated in a shorter time than with the Mickle disintegrator.

The cell walls were digested with trypsin or chymotrypsin by suspending up to 500 mg in 125 to 250 ml of 0.066 M, pH 7.8 phosphate buffer and adding 2 mg of crystalline enzyme. After 24 hr at 37 C, the residual cell walls were sedimented by centrifugation and resuspended in fresh buffer containing additional enzyme. After another 24 to 48 hr, the cell walls were again sedimented by centrifugation, washed once with phosphate buffer, twice with distilled water, and finally suspended in a small amount of distilled water. The suspension was lyophilized and stored.

Polysaccharide preparations were obtained by the method of McCarty (1952*b*). The resulting products were colored and were further purified in some cases by dissolving in 40 per cent ethanol

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and treating the ethanolic solutions with a small amount of Darco G60. In one case, a C polysaccharide preparation was passed through a Darco G60-Celite column (Whistler and Durso, 1950) and eluted with aqueous ethanol solutions of increasing ethanol concentration. The column was prewashed with dilute HCl, 95 per cent ethanol, and water. After the C polysaccharide in aqueous solution had been adsorbed, the column was washed with 3 L of water and 3 L of 20 per cent ethanol. The bulk of the C polysaccharide recovered was obtained by elution with 35 per cent ethanol.

The procedures described above were also applied to cultures of a group A variant streptococcus (no. K43-Var-C) obtained from Dr. Maclyn McCarty, Rockefeller Institute for Medical Research. The cell walls and polysaccharide from this organism have been studied by McCarty and Lancefield (1955) and differ from the group A streptococci chiefly in higher ratios of rhamnose to glucosamine, approximately 5:1 as compared to 3:2, and in the specific serological reactivity of the polysaccharide.

Hydrolysis and chromatographic analysis. The hydrolysis of these samples prior to analysis was essentially the method of Carsten and Kabat (1956). Samples for analysis were first dried over P_2O_5, *in vacuo*, for 24 hr. They were then suspended or dissolved in 100 ml of 1 N HCl and refluxed for 36 hr. The clear solutions were concentrated to dryness under reduced pressure, a small amount of water was added to the dry residue, and the solution was again concentrated to dryness. Several repetitions of the latter procedure removed almost all of the acid. The

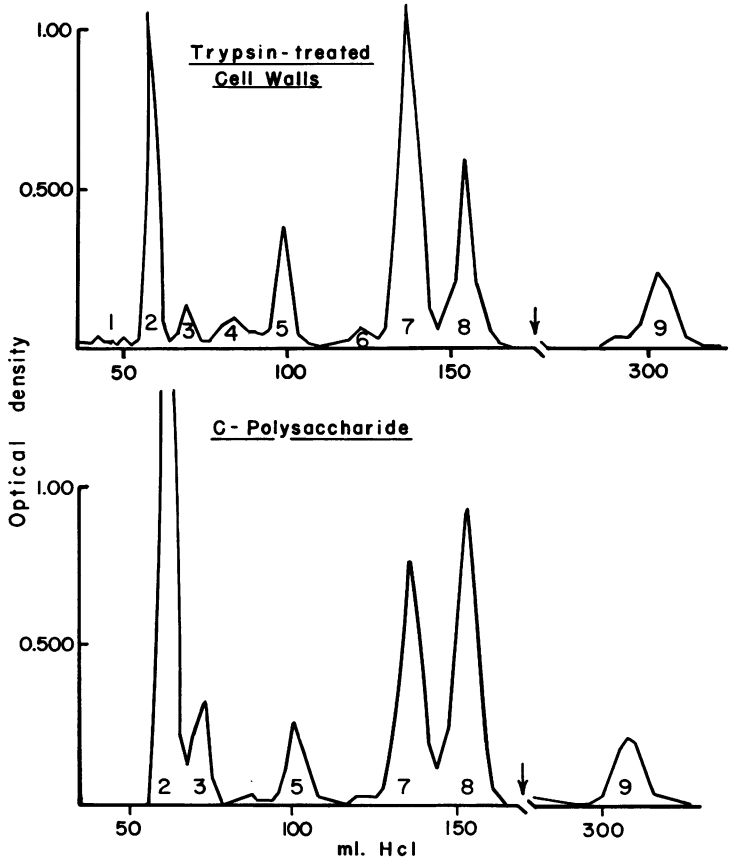

Figure 1. Elution diagrams of hydrolyzates of cell walls and C polysaccharide. Dowex 50-X8 columns (1 by 55 cm) eluted with 1.5 N HCl and then with 3.5 N HCl from the point indicated by the arrow. The effluent was analyzed by the quantitative ninhydrin method. The major components are: *2*, glucosamine; *3*, muramic acid; *5*, glutamic acid, *7*, alanine; *8*, ammonia; *9*, lysine. Peak *6* is glycine; *1* and *4* are mixtures.

syrupy residue was dissolved in water and quantitatively transferred to a volumetric flask; aliquots of this solution were analyzed.

Dowex 50-X8 (H form) purified by the method of Moore and Stein (1951) was used to form a column 1 by 55 cm. After conversion to the hydrogen form, the sample was adsorbed on the column and then washed with 50 ml of water. Elution was begun with a 10 cm head of water above the resin bed. Dropwise addition of 1.5 N HCl to the column gave an initial water-to-HCl gradient which extended over the first 30 tubes, and resulted in cleaner separation of glucosamine and muramic acid. After emergence of the ammonia peak, elution was continued with 3.5 N HCl until the lysine peak emerged. The 1 ml effluent fractions were analyzed by the quantitative ninhydrin method of Moore and Stein (1954). The peaks were identified by paper chromatography.

Chemical methods. Hexosamine analyses followed the methods of Boas (1953), Dische and Borenfreund (1950), and Roseman and Daffner's modification (1956) of the Boas method. L-Rhamnose was analyzed by the method of Dische and Shettles (1948). N-acetylhexosamine was analyzed by the methods of Reissig *et al.* (1955) and Aminoff *et al.* (1952).

Muramic acid was identified following isolation from an acid hydrolyzate of trypsin-treated cell walls. Five hundred mg of trypsin-treated cell walls were hydrolyzed and passed through a Dowex 50 column (2.5 by 70 cm). Elution was carried out as described above and the muramic acid fraction isolated. This fraction was again chromatographed on a Dowex 50 column (1 by 50 cm) and separated from a small amount of glucosamine. Unfortunately, authentic samples of muramic acid were not available for comparison, but the criteria cited below indicated the identity of peak 3 (figure 1) with this compound.

RESULTS

Typical elution diagrams of a sample of hydrolyzed, trypsin-treated cell walls and of a C polysaccharide sample are shown in figure 1. Peak 1 was found to give at least three spots on chromatograms sprayed with ninhydrin and two additional spots after hydrolysis in 2 N HCl. Peaks 2 and 3 are hexosamines, number 2 representing glucosamine and 3, muramic acid. Peak 4 was not homogeneous and contained small amounts of serine, threonine, and one other ninhydrin-reactive substance. The other peaks were identified as follows: peak 5, glutamic acid; 6, glycine; 7, alanine; 8, ammonia; and 9, lysine. Since the total amount of the minor components was small (less than 10 per cent of the total amino acids) they were not characterized further. However, characterization and quantitative analysis of all the amino acids of cell walls, trypsin-treated cell walls, C polysaccharide, and M protein is in progress in this laboratory at the present time and the results will be published in a separate paper.

The identification of peak 3 as muramic acid was verified by the following comparisons with published data: (1) the position of elution relative to glucosamine hydrochloride from a Dowex 50 column eluted with HCl; (2) paper chromatograms employing the solvent systems used by Park (1952); (3) the characteristic orange color and the pronounced absorption maximum at 510 mμ of its chromogen in the Elson-Morgan reaction (Strange and Powell, 1954); (4) the greater amount of color obtained from N-acetylmuramic acid than from an equimolar amount of N-acetyl glucosamine in the Morgan-Elson reaction

TABLE 1

Comparison of muramic acid and unknown compound

1. Mobility on paper chromatograms:

Solvents[a]	*Rf muramic acid*[a]	*Rf unknown*
75% phenol	0.54	0.57
BuOH:HOAc:H_2O	0.52	0.55
t-Amyl alcohol: formic acid:H_2O	0.47	0.52

2. Ratios of color produced per mole by N-acetylated sugars in Morgan-Elson test (relative to N-acetyl glucosamine)[b, c]:

Muramic acid	*Unknown*
2–3	2.9

3. Same orange color produced in Elson-Morgan test; chromogen possessing absorption maximum at 510 mμ.[d]
4. Eluted from Dowex 50 in same position relative to glucosamine hydrochloride.[e]
5. Spectrum produced in Dische and Borenfreund test the same as glucosamine.[d]

[a] Park, 1952.
[b] Aminoff *et al.*, 1952.
[c] Reissig *et al.*, 1955.
[d] Strange and Powell, 1954.
[e] Strange and Dark, 1956.

TABLE 2

Analyses of cell walls after treatment with chymotrypsin

Compound	Per Cent Dry Weight*	Mole Ratio
Glutamic acid	12	1.0
Lysine	11	1.0
Alanine	30	3.9
Glucosamine	24	1.6
Rhamnose	22	1.4

* The per cent weights refer to the mg of substance liberated from 100 mg of chymotrypsin-treated cell walls.

TABLE 3

Analyses of enzymatically obtained C polysaccharide

Polysaccharide Preparation	Compound	Per Cent Dry Weight*	Mole Ratio
Crude	Glutamic acid	6	1.0
	Lysine	—	—
	Alanine	15	3.8
	Glucosamine	45	5.6
	Rhamnose	48	6.1
Charcoal-treated	Glutamic acid	3	1.0
	Lysine	3	1.1
	Alanine	7	4.2
	Glucosamine	40	12.0
	Rhamnose	54	16.0

* The mg of substance liberated from 100 mg of polysaccharide.

(Aminoff *et al.*, 1952) (Reissig *et al.*, 1955). The results leading to the identification of muramic acid are summarized in table 1.

The results of a typical analysis of enzyme-treated cell walls are summarized in table 2. There are no differences in the results obtained with trypsin- and with chymotrypsin-treated cell walls. With the moles of glutamic acid taken as one, the mole ratios of the other amino acids are four for alanine and one for lysine. It is apparent that the bulk of the enzyme-treated cell wall is made up of polysaccharide and simple polypeptides.

The mole ratio for lysine is 1.0 in this determination. A few of the other determinations showed lower ratios for lysine, but this is ascribed to the decomposition of lysine, which is known to occur under the hydrolysis conditions used here (Carsten and Kabat, 1956).

The analyses of various polysaccharide samples are summarized in table 3. The values for the charcoal-treated sample are the averages of three analyses. Of the three samples analyzed, two were treated with charcoal in a batch process and one was purified on a charcoal column. No significant differences were found in the products from the two processes.

The polysaccharide preparations obtained following charcoal treatment contain approximately half the amount of amino acids found in the untreated polysaccharide, resulting in a relative enrichment in the carbohydrate portion. There is, however, no change evident in the molar ratios of the amino acids in the polysaccharide before and after charcoal treatment. A comparison of the molar ratios of the amino acids in the cell wall (table 2) and in the polysaccharide (table 3) reveals a striking similarity. In each case, when glutamic acid is taken as one, lysine is one, and alanine is four. It appears from these data that the polysaccharide products obtained after enzymatic hydrolysis of the cell wall have a smaller content of amino acids than the cell wall, but these are retained in the proportions found in the cell wall. Further treatment of the polysaccharide with charcoal removes those fractions of the polysaccharide preparation which are relatively richer in amino acid content but the molar ratios remain unchanged.

The application of the methods of analysis already detailed to a variant streptococcus are shown in table 4. The analyses of McCarty and Lancefield (1955) indicated that this variant

TABLE 4

Results of analyses of K variant cell walls and variant polysaccharide

Preparation	Compound	Per Cent Dry Weight*	Mole Ratio
Trypsin-treated cell walls	Glutamic acid	8	1.0
	Lysine	6	0.8
	Alanine	16	3.6
	Glucosamine	18	0.84
	Rhamnose	40	4.1
Polysaccharide (crude)	Glutamic acid	7	1.0
	Lysine	8	1.2
	Alanine	14	4.0
	Glucosamine	13	1.4
	Rhamnose	61	7.5

* The mg of substance obtained from 100 mg of material hydrolyzed.

TABLE 5

Mole ratios of components of the cell walls of Staphylococcus aureus and Streptococcus pyogenes

Compound	*S. aureus**	*S. pyogenes*
Glutamic acid...	1.0	1.0
Lysine.....	1.17	1.0
Alanine.....	3.5	3.9
Hexosamine.....	0.93	1.6

* Park and Strominger, 1957.

differed from group A streptococci not only in group reactivity, but in the relative amounts of rhamnose and glucosamine in the cell wall. They showed that in the variant cell walls, as well as the polysaccharide obtained from those walls, the ratio of rhamnose to glucosamine was approximately 5, in contrast to a ratio of approximately 2 in group A organisms. The analyses reported here show a ratio for the variant of 5.0 and the percentages of rhamnose and glucosamine found are in good agreement with the results of those workers.

As in the type 14 group A streptococci already discussed, the group A variant cell walls show amino acid ratios of one lysine and four alanines for each glutamic acid. The variant polysaccharide also contains the same mole ratios of the amino acids as the cell wall. A point of difference between the two strains is that the variant cell wall loses only 32 per cent of its dry weight on treatment with trypsin, whereas the group A strains in this laboratory always show a loss of 40 per cent in weight.

DISCUSSION

The experiments summarized here show that the major components of the enzyme-treated cell walls of group A hemolytic streptococci are L-rhamnose, glucosamine, alanine, lysine, glutamic acid, and muramic acid, a result which is in good agreement with the semiquantitative results of Cummins and Harris (1956). The latter authors concluded that this amino acid composition was characteristic of most gram-positive organisms. The close correspondence of the ratios of the amino acids which have been obtained by quantitative determinations on the cell walls of *S. aureus* (Park and Strominger, 1957) with the analyses of group A streptococcal cell walls reported here lends further support to this conclusion (see table 5).

There is no difference apparent in the composition of cell walls obtained by treatment with trypsin, chymotrypsin, or crude mixtures of pancreatic enzymes. In all of these treatments, the gross effect was a loss of 40 per cent in the dry weight of the cell walls, with little change in their appearance, as shown by the electron microscope. The work of Strange and Thorne (1957), of Salton and Ghuysen (1957), of Park and Strominger (1957), and of Snell *et al.* (1955) has indicated that the D-isomers of alanine and glutamic acid are present in the cell walls of bacteria in large amounts. A structure containing D-amino acids might be resistant to hydrolysis by enzymes such as trypsin, chymotrypsin, pepsin, or even the combination of enzymes found in crude pancreatic extracts. This resistance to hydrolysis may explain the similarity in products obtained with different enzymes (Cummins and Harris, 1956).

The cell walls of the variant organism showed a smaller decrease in dry weight when treated with chymotrypsin. However, the same ratios of glutamic acid to lysine to alanine of 1:1:4 are found in variant streptococcal cell walls as in the group A cell walls, indicating a fundamental similarity in these streptococcal cell walls.

The glucosamine of the cell wall may arise in part from muramic acid. The method of hydrolysis used here seems to hydrolyze most of the muramic acid to glucosamine, so that it is not certain whether or not the small amount of muramic acid in the acid hydrolyzates represents any substantial portion of that which is in the intact cell wall. Preliminary experiments have indicated, however, that after ½ hr of hydrolysis at 100 C in 0.1 N HCl, there is about twice as much N-acetylglucosamine as N-acetylmuramic acid present in the hydrolyzates. After further hydrolysis, the ratio of glucosamine to muramic acid is still slightly greater than two, and it seems that at least a third of the hexosamine of the cell wall is in the form of muramic acid.

The C polysaccharide of streptococcal cell walls which makes up 50 to 60 per cent of the dry weight of trypsin-treated cell walls may be obtained apparently free of any protein residue by formamide extracts of whole cells (Zittle and Harris, 1942). A more interesting preparation in terms of defining structure is the polysaccharide preparation recovered after lysing cell walls with extracellular enzymes present in culture filtrates of *Streptomyces albus* (McCarty,

1952*a*). McCarty (1952*b*) studied C polysaccharide obtained in this manner and found that approximately 85 per cent of this preparation consisted of rhamnose and hexosamine. Amino acids apparently comprised the balance since the nitrogen content was higher than could be accounted for by the hexosamine present.

The present analyses (table 3) of various enzymatically obtained C polysaccharide preparations showed the same amino acids, in the same molar ratios, as in the cell walls. Table 3 also shows that the glucosamine:glutamic acid ratio in the polysaccharide is considerably higher than in the wall. Thus, in the process of release of the polysaccharide from the cell wall by enzymes present in the mold filtrates, most of the linkages between the peptide moiety and the polysaccharide are broken. Activities other than saccharase have been demonstrated in the mold filtrates, as, for example, the evidence for at least two peptidases which was presented by Salton and Ghuysen (1957).

The differences in the charcoal-treated and crude polysaccharide preparations show that the C polysaccharide preparations are not homogeneous, but consist of a variety of large molecules composed of varying amounts of carbohydrate and amino acids. McCarty (1952*b*) compared the rhamnose to glucosamine ratios of fractions precipitated at different concentrations of ethanol and found that the ratios differed, indicating the heterogeneity of the preparations. After charcoal adsorption of the polysaccharide, the recovered material consisted of 3 per cent glutamic acid and 7 per cent alanine, as compared to 6 and 15 per cent of those compounds in the crude preparations, indicating again that the crude C polysaccharide preparations are heterogeneous.

From the data given in this paper and from the work of other investigators, (McCarty, 1956; Park and Strominger, 1957) there seems to be a consistent and simple structure which may be envisioned for the structure of the streptococcal cell wall. It includes a relatively large variety of amino acids, and is in that respect dissimilar to most other gram-positive organisms (Salton, 1953). Upon treatment with proteolytic enzymes, there is a 40 per cent loss in dry weight and complete loss of the type-specific M serological activity (Barkulis and Jones, 1957). The insoluble residue which remains after the protease treatment has a composition characteristic of the cell walls of gram-positive organisms and consists of carbohydrate and repeating peptide units made up of lysine, alanine, and glutamic acid. These peptide chains may then be attached to an N-acetylated hexosamine through a carboxyethyl group in accord with the findings of Strange (1956) and the formulations of Park and Strominger (1957), and the hexosamine-peptide units in turn bound to a rhamnose polysaccharide through a glycosidic linkage from the hexosamine to the polysaccharide (McCarty, 1956).

The growing evidence for high concentrations of glutamic acid, alanine, and lysine (with diaminopimelic acid substituting for lysine in some species) in the cell wall structure of gram-positive organisms suggests that these amino acids linked to hexosamine compose the basic structure of the cell walls of gram-positive organisms. In the case of *S. aureus*, in which no other sugar component is found, the hexosamine portions of each hexosamine-peptide unit may be linked to each other. In species which contain other sugars, cell wall components, such as the rhamnose polysaccharide of hemolytic streptococci, the hexosamine-amino acid units may be attached to this polysaccharide rather than to each other. The peptide chains of these repeating units may also be attached to other amino acids which are readily removed by proteolytic enzymes. In the group A hemolytic streptococci, the additional amino acids almost double the weight of the basic cell wall structure and are the principal components of the M protein which defines type specificity in this group.

SUMMARY

Quantitative amino acid analyses have been made of trypsin-treated, type 14, group A, hemolytic streptococcal cell walls and of enzymatically liberated polysaccharide from the walls. The main components obtained after acid hydrolysis are: glucosamine, rhamnose, glutamic acid, lysine, alanine, and a small amount of muramic acid. The cell walls showed molar ratios of glutamic acid:lysine:alanine:glucosamine:rhamnose to be approximately 1:1:4:1.6:1.4. The enzymatically released C polysaccharide of the cell walls showed the ratios 1:1:4:6:6. Charcoal treatment of the polysaccharide resulted in a product with the ratios 1:1:4:12:16, indicating a preferential removal of amino acid rich portions of the polysaccharide preparations by the charcoal. The trypsin-treated cell walls of a variant group A

streptococcus showed the following ratios; 1:1:4.0:0.84:4.1; the corresponding polysaccharide gave the results 1:1:4:1.4:7.5.

It is concluded from these data that the structural elements of the streptococcal cell wall are composed of polysaccharide linked to a polypeptide composed of glutamic acid, lysine and alanine. The peptide chains are composed of a series of unit hexapeptides, each unit containing one glutamic acid, one lysine, and four alanines in an as yet undetermined sequence.

The molar ratios obtained for the streptococcal cell wall are the same as those obtained for the staphylococcal cell wall, and are probably similar to those of other gram-positive organisms.

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