STUDIES OF STREPTOCOCCAL CELL WALLS

I. Isolation, Chemical Composition, and Preparation of M Protein[1]

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The emergence of techniques for the isolation of cell walls of microorganisms (Dawson, 1949; Salton and Horne, 1951) has opened new approaches to immunological and physiological problems presented by the microbial surface. The recovery of clean cell walls of the group A hemolytic streptococci in good yield particularly enhances the study of such surface antigens as the group C polysaccharides and the type specific M proteins. Cell walls present a more advantageous starting material than the whole organisms for the purification and characterization of these substances. In this paper, the preparation of cell walls from type 14, group A hemolytic streptococci using the Mickle disintegrator, is described, and data of the chemical composition are presented. The extraction and purification of type 14 M protein from cell walls by the procedure of Lancefield and Perlmann (1952) is also detailed.

MATERIALS AND METHODS

Growth of the organism and preparation of cell walls. Three cultures of group A, type 14 hemolytic streptococci were used. Two of the cultures had been isolated from patients with respiratory infections at the Streptococcal Disease Laboratory, Warren Air Force Base, Wyoming, and were made available to us by Dr. E. N. Fox. The third culture, designated S-23, was obtained from Dr. R. C. Lancefield. The organisms were grown either in Tod-Hewitt broth or in a medium containing autoclaved brain heart infusion (Difco) in a final concentration of 3.7 per cent and a solution of salts sterilized by filtration in the following concentrations: glucose, 1 per cent; $NaHCO_3$, 0.8 per cent; $NaH_2PO_4 \cdot H_2O$, 0.05 per cent; $Na_2HPO_4 \cdot 7H_2O$, 0.22 per cent. The organisms were harvested from 12–16 hr cultures and washed twice in distilled water. The conditions defined by Salton and Horne (1951) for cell breakage in the Mickle disintegrator were found suitable for these organisms and were used with minor variations. Each gram of cells (wet wt) packed by centrifugation at 4,000 rpm was suspended in 10 ml of distilled water. Five milliliter portions of this cell suspension were mixed with an equal volume of Ballotini no. 12 glass beads in rubber stoppered glass vials of 20 ml volume. Two such vials were vibrated on the Mickle disintegrator for 20 min. The vibrating forks of the instrument were set to obtain maximal amplitude. The time and amplitude of vibration were found to be critical in order to obtain disruption of the greatest number of cells without appreciable fragmentation of the cell walls. The glass beads were recovered by filtering the mixture through a coarse fritted glass funnel. The cell walls were sedimented by centrifugation at 10,000 rpm in a high speed angle head centrifuge (Servall, SS-1). They were washed six times by resuspension in water or phosphate buffer as specified, and resedimented in the centrifuge. Aliquots of the washed cell walls were then studied by electron or phase microscopy to determine the completeness of cell breakage. The experiments reported are from preparations containing approximately 0.1 per cent of whole cells. Figures 1 to 3 are from typical preparations of cell walls and illustrate the ease with which they are differentiated from whole cells in the electron microscope. The adequacy of the washing procedure could also be gauged by the amounts of small, spherical, electron dense particles which were present and probably cytoplasmic in origin. These particles decreased with each wash and the final suspensions of cell walls were relatively free of them.

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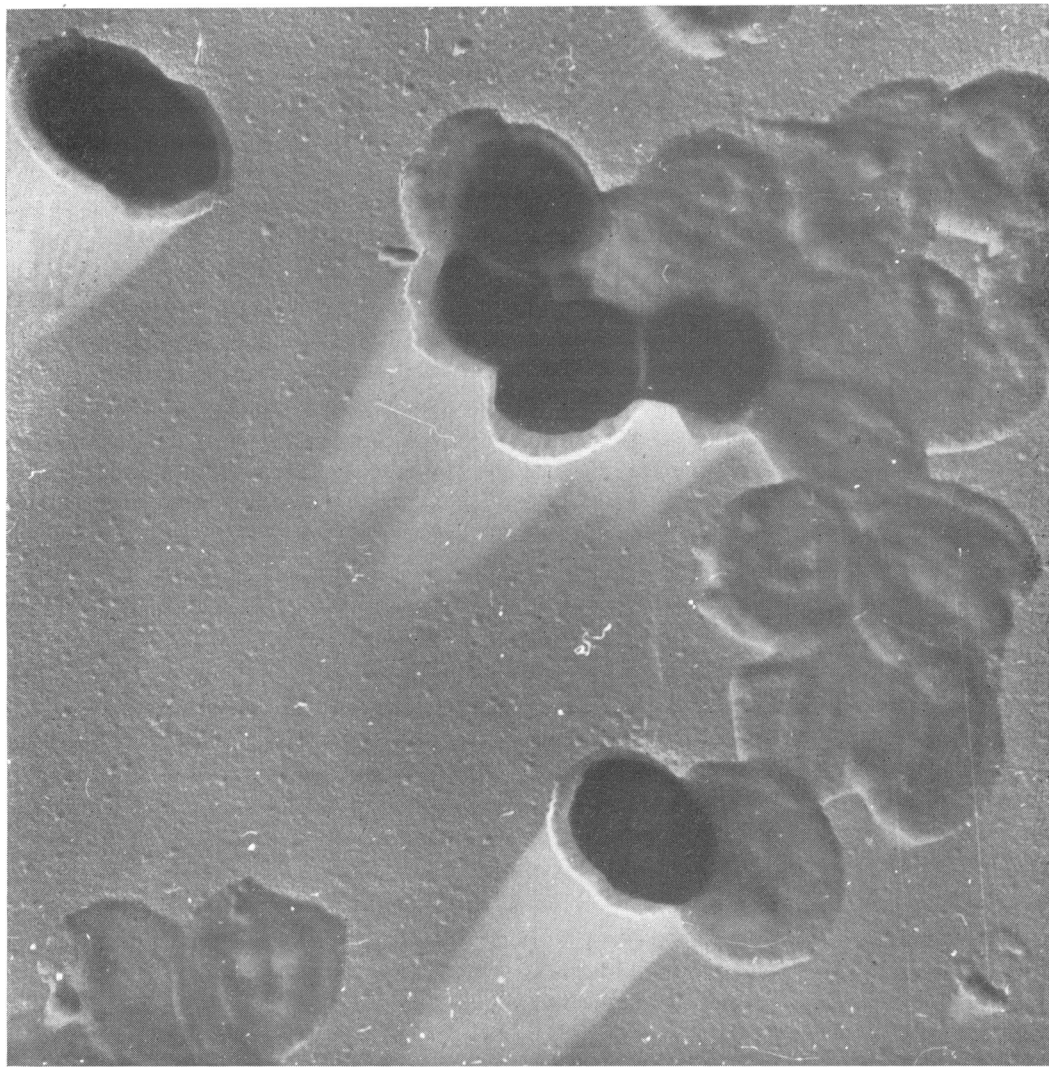

Figure 1. Electron micrograph of a washed suspension of whole cells and cell walls of type 14, group A streptococcus after 10 min vibration on the Mickle disintegrator. The preparation was shadowed with chromium at an angle of 15 degrees. × 20,000 magnification. The whole cells appear opaque and cast long shadows as compared to the cell walls.

When cell walls were exposed to ribonuclease or trypsin (Armour and Co.) a final concentration of 0.01 mg/ml of the crystalline enzyme was employed and the digesting suspension was dialyzed against 0.01 M potassium phosphate buffer at pH 7.8 for 16 hr at room temperature. Chloroform was added to inhibit growth of microbial contaminants. The cell walls were then recovered by centrifugation and washed three times with distilled water unless otherwise specified.

Preparation of cell wall extracts. Cell walls were extracted by adjusting the pH of suspensions to 2 with N HCl and immersing in a boiling water bath for 10 min (Lancefield, 1928). The tubes were then cooled and the pH was adjusted to 7.5 with N NaOH. The walls were sedimented in the centrifuge and the clear, colorless supernatants decanted. This procedure does not alter microscopic appearance of cell walls except for a decrease in opacity.

Serology. The content of M protein and C poly-

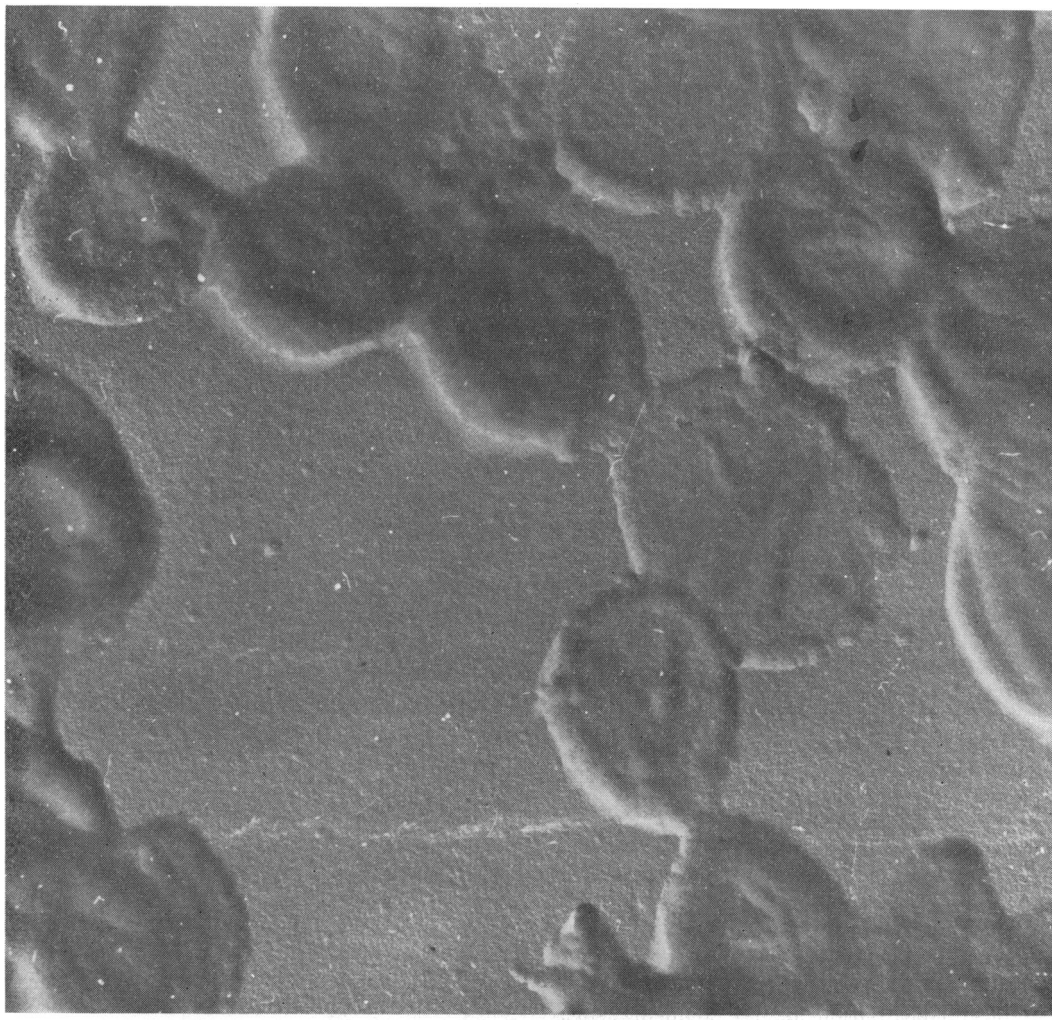

Figure 2. Electron micrograph of washed cell walls of type 14, group A streptococcus obtained after 20 min vibration on the Mickle disintegrator. The preparation was shadowed as in figure 1. × 30,000 magnification.

saccharide in extracts and in ammonium sulfate fractions was roughly assayed by the ring test. Type 14 anti-M sera were obtained from rabbits immunized according to the procedure of Lancefield (1947) with heat killed streptococci. The sera were absorbed twice with acid-heat extracts of type 24 organisms. Five hundred micrograms of type 24 dried extract were added per ml of serum in the first absorption and 250 μg/ml in the second. The sera were incubated at 37 C for 2 hr and then placed in the cold room at 4 C for 4 days. The precipitates formed during each adsorption were removed by centrifugation. Group A antisera were obtained from rabbits immunized with heat killed type 14 cells which had been previously treated with trypsin to remove the M protein. They gave positive ring tests with solutions containing as little as 0.5 μg purified C polysaccharide per ml.

Chemical. Cell walls were hydrolyzed in 2 N HCl at 100 C for 2 hr in sealed ampoules. The hydrolyzates were dried *in vacuo* to remove HCl, redissolved in water, and carbohydrate components identified by paper chromatography, using the ethyl acetate:pyridine:water solvent system described by Jermyn and Isherwood (1949). The papers were developed by spraying with aniline phthalate in water saturated butanol. Reducing

Figure 3. Higher magnification, × 67,000, of cell walls described in figure 2

sugars were determined on aliquots of the hydrolyzates by the method of Nelson (1944). On an equal molar basis, rhamnose and hexosamine standards gave 49 and 86 per cent of the color development obtained with glucose. Rhamnose was assayed by the cysteine-sulfuric procedure of Dische and Shettles (1948). By subtracting the rhamnose contribution to the reducing sugar value obtained for a hydrolyzate, the hexosamine content could be calculated. The validity of this method was established by determinations on known rhamnose-hexosamine mixtures of varying composition. Nitrogen was determined by the micro-Kjeldahl method. The protein content of extracts and ammonium sulfate fractions was determined by the method of Sutherland *et al.* (1949).

RESULTS

The binding of nucleic acid by cell walls. The presence of a nucleic acid or nucleoprotein component of cell walls was sought in early experiments by measuring the ultraviolet absorption spectra of aqueous suspensions. Salton and Horne (1951) cited the absence of an absorption peak at 260 mμ in cell wall suspensions of *Streptococcus*

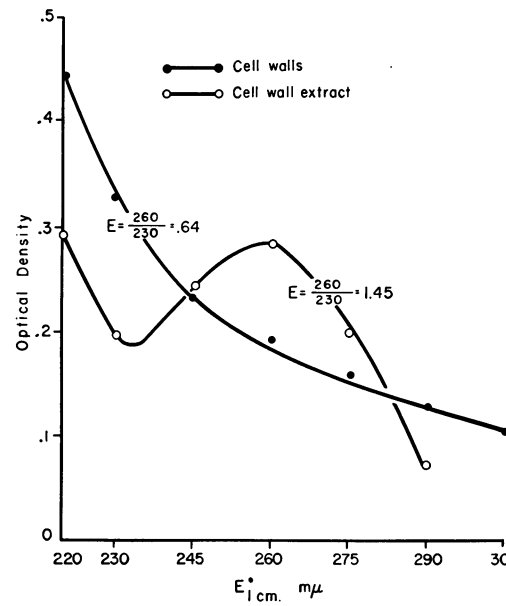

Figure 4. A comparison of the ultraviolet absorption spectrum of a suspension and an extract of cell walls.

* Cuvettes contained in a 3 ml volume approximately 0.1 mg cell walls or the extract from 2.5 mg (dry wt).

TABLE 1

Effect of pH, ribonuclease and trypsin on the adsorption of nucleic acids by cell walls

Experiment	Extracts of Untreated Cell Walls		Extracts of Ribonuclease Exposed Cell Walls		Extracts of Trypsin Exposed Cell Walls	
	$E^{*}_{1\,cm}$ 260 mμ	$\frac{E^{*}_{1\,cm}}{\frac{260}{230}}$ mμ	$E^{*}_{1\,cm}$ 260 mμ	$\frac{E^{*}_{1\,cm}}{\frac{260}{230}}$ mμ	$E^{*}_{1\,cm}$ 260 mμ	$\frac{E^{*}_{1\,cm}}{\frac{260}{230}}$ mμ
I.						
No exposure to nucleic acids	0.197	1.14	0.058	0.50	0.169	1.01
Exposed to nucleic acids at pH 7.4	0.191	0.97	0.154	0.73	0.257	1.55
Exposed to nucleic acids at pH 6.4	0.210	1.13	0.200	1.17	0.295	1.52
Exposed to nucleic acids at pH 5.4	0.249	1.21	0.255	1.37	0.273	1.63
II.						
No exposure to nucleic acids	0.328	1.52	0.048	0.26	0.110	0.71
Exposed to nucleic acids at pH 7.4	0.229	1.37	0.109	0.64	0.121	0.69
Exposed to nucleic acids at pH 6.4	0.316	1.26	0.195	0.73	0.193	0.78
Exposed to nucleic acids at pH 5.4	0.344	1.50	0.348	1.30	0.406	1.63

* Concentration of extract is the amount obtained in 3 ml by acid-heat treatment of 2.5 mg cell walls (dry wt).

faecalis as supporting evidence for the absence of nucleic acid constituents. Similar spectra were obtained with most suspensions of water washed cell walls of the organisms used in this study. However, the absorption spectra of acid-heat extracts of these cell walls were invariably characterized by a marked peak at 260 mμ. This is illustrated by the ultraviolet absorption curves of a cell wall suspension and its extract shown in figure 4. It is evident that spectrophotometric determinations on cell wall suspensions cannot be employed to evaluate the amount, or even the presence of nucleic acid components.

During the course of these experiments, increasing optical densities at 260 mμ were observed with some cell wall suspensions which were allowed to stand for several hours at room temperature. This suggested that variable amounts of nucleic acid were adsorbed onto cell walls and could be detected in the spectrophotometer in proportion to the extent of elution. The ability of cell walls to bind nucleic acids at varying pH was therefore investigated in the following manner: Twenty milligram amounts (dried wt) of water washed cell walls were suspended in vol of 5 ml containing 0.001 M $MgCl_2$, and 10 mg of yeast nucleic acid in 0.1 M phosphate buffer at a pH of 5.4, 6.4, or 7.4. The concentration of nucleic acid used was based on an approximation of the amount which would be released in the process of disintegrating whole cells under the conditions specified. The suspensions were agitated on a mechanical shaker for 30 min at room temperature. The cell walls were recovered by centrifugation and washed three times with distilled water. An aliquot was then extracted in the usual manner. A single extraction was found adequate to remove all of the 260 mμ absorbing material. The same procedure was carried out concomitantly on cell walls which had been previously treated with ribonuclease or trypsin. Ultraviolet absorption spectra of the extracts were then taken. In order to express the results of the spectrophotometric data in tabular form the optical density at 260 mμ and the ratio of that value to the optical density measured at 230 mμ are recorded in table 1. The 260/230 ratios observed with spectra which have markedly different 260 mμ absorption characteristics are illustrated in figure 4. With the concentrations of extract used, absence of nucleic acid was evidenced by spectra which showed a continual decrease in optical density from 220 to 300 mμ and were characterized by 260/230 ratios of 0.5 or less and optical densities of 0.05 or less at 260 mμ. Therefore, although it was not possible to demonstrate spectrophotometrically the nucleic acid content of cell walls in suspension, a comparison of the relative amounts of nucleic acid in their extracts could be made.

It is apparent from the data in table 1 that the amount of nucleic acid extracted from cell walls

is related to the pH at which they were exposed. Some loss of the nucleic acid initially absorbed on cell walls during Mickle disintegration was encountered on re-exposure to nucleic acid at pH 7.4. As the pH was decreased to 5.4, this was recovered. Extracts of ribonuclease treated cell walls contained little, if any, nucleic acid. However, they reabsorbed increasing amounts of nucleic acid as the pH was decreased. Extracts of trypsin treated walls showed a variable decrease in nucleic acid. Trypsinized cell walls were also capable of rebinding nucleic acid and at pH 5.4 the amounts taken up usually exceeded those observed with nontrypsinized walls. These findings strengthened the view that the 260 mμ absorbing material found in extracts from cell walls resulted from an ionic binding of a portion of the intracellular nucleic acid freed during cell breakage and that this was not a component of the cell wall. Sufficient washing of cell walls in phosphate buffer at neutral pH should, therefore, displace such adherents into solution. This was achieved, as shown in table 2, by three washes in 0.1 M phosphate buffer which eliminated 260 mμ absorbing material as effectively as did treatment of cell walls with ribonuclease. Cell walls employed in all subsequent studies were routinely washed 3 times in water, followed by 3 washings in 0.1 M phosphate buffer at pH 7.4.

TABLE 2

Comparison of ribonuclease digestion and washing with phosphate buffer on the elimination of 260 mμ absorbing materials from cell walls

	Extracts from Cell Walls after Treatment Specified	
	$E^{*}_{1\,cm}$ 260 mμ	$\frac{E^{*}_{1cm}}{\frac{260}{230}}$ mμ
Cell walls washed 3 times with distilled water at pH 7.0	0.141	1.15
Cell walls washed 3 times with 0.1 m phosphate buffer at pH 7.0	0.048	0.29
Cell walls exposed to ribonuclease and then washed 3 times with distilled water	0.054	0.31

* Concentrations as specified in table 1.

TABLE 3

Chemical composition of type 14, group A streptococcal cell walls

	Per Cent of Dry Weight
Cell walls	
Fraction of whole cells	23–25
Nitrogen	9.5–10
Rhamnose + hexosamine	34–36
Rhamnose	21–22
Protein	64–66
Cell walls after trypsin exposure	
Fraction not solubilized	59–61
Nitrogen	7.5
Rhamnose + hexosamine	60–63
Protein	37–40

Chemical composition of the cell wall. Table 3 summarizes the results of many determinations on type 14 streptococcal cell walls before and after trypsin exposure. This entity makes up an appreciable portion, 23 to 25 per cent, of the weight of these organisms. Capsular material did not pose a problem since it is not present on cells harvested after 12 to 16 hr growth. Chromatography of cell wall hydrolyzates revealed the presence of two monosaccharides, L-rhamnose[2] and hexosamine. The latter is acetylated, at least in part, as evidenced by a strong acetyl hexosamine spot in chromatograms of partial hydrolyzates. The sugars are the principal components of the C polysaccharide (McCarty, 1952; Schmidt, 1952), which in turn makes up slightly over a third of the cell wall. The ratio of rhamnose to hexosamine is approximately 3:2 on a molar basis.

Since no evidence for a lipid fraction has been found by extraction with a variety of lipid solvents, the remainder of the cell wall has been somewhat arbitrarily called protein. Work currently in progress to determine quantitatively the amino acids which are present should permit a more accurate assessment of this fraction. Incubation with trypsin solubilizes approximately 40 per cent of the cell walls at the expense of the protein component, two-thirds of which is ren-

[2] We are indebted to Dr. Ellis Englesberg for verifying the configuration of a sample of cell wall rhamnose by assay with an L-rhamnose isomerase from a mutant of *Pasteurella pestis* (Englesberg, 1955), and to Dr. Michael Doudoroff for confirming the absence of D-rhamnose in a duplicate sample by assay with a mannose isomerase from *Pseudomonas saccharophila*, which is specific for the D-isomer (Palleroni and Doudoroff, 1956).

dered dialyzable. The M protein is lost by this treatment, but the appearance of cell walls under the electron microscope is not altered. The structural integrity of the cell wall is thus seen to be derived from material which is principally carbohydrate (60 to 63 per cent) in composition, and is highly resistant to chemical solution or modification. Heating in alkali, acid, formamide (Fuller, 1938), or urea will result in varying degrees of hydrolysis. Complete solution has been obtained recently with cold anhydrous hydroflouric acid in experiments which will be reported separately. Enzymatic cleavage and release of C polysaccharide has been brought about by the combined action of trypsin and an enzyme obtained from the culture filtrates of *Streptomyces albus* (Maxted, 1948; McCarty, 1952).

The preparation of M protein from cell walls. Lancefield and Perlmann (1952) have described the preparation of M protein from type 1, group A hemolytic streptococci. Their procedure, with minor modifications, was followed in obtaining M protein from cell walls of the type 14 organisms. The following is a typical protocol: Thirty-two grams (packed wet wt) of S-23 cells were harvested from nine 1-L cultures by Sharples centrifugation. After two water washes, the cells were suspended in distilled water, broken in the Mickle disintegrator, isolated and washed as described. If the washing procedure is carefully performed so that aggregates are finely dispersed during each suspension, nucleic acid is removed. Incubation of the washed cell walls with ribonuclease can also be used to insure removal of nucleic acids.

The washed cell walls, approx 1,050 mg dry wt, were then suspended in 100 ml of distilled water and extracted. The extraction was repeated a second time and the two extracts were pooled and cooled to 4 C. Subsequent operations were carried out in the cold room. Solid ammonium sulfate was added gradually with continuous stirring until a saturation of 0.3 was reached (based on full saturation as 70 g/100 ml). The precipitate which formed was sedimented by centrifugation and the supernatant decanted. The pellet was resuspended in 0.01 M potassium phosphate at pH 7.4, but was only partially soluble. The insoluble portion was sedimented in the centrifuge and discarded. The supernatant was saved and labelled fraction S-1.

To the 0.3 saturated ammonium sulfate supernatant, additional salt was added until 0.6 saturation was attained. The copious, white precipitate which formed was recovered by centrifugation and was completely redissolved in phosphate buffer (fraction S-2). The 0.6 saturated supernatant was designated fraction S-3. The three fractions were dialyzed against several changes of 0.001 M potassium phosphate pH 7.4 for 24 hr.

The fractions were assayed for protein and rhamnose. Serologic reactivity was determined on dilutions which contained equal amounts of protein. The findings are summarized in table 4. The extraction procedure solubilized approx 13 per cent of the protein and 27 per cent of the rhamnose originally present in the cell walls. The rhamnose was not dialyzable and was apparently in the form of C polysaccharide as shown by the excellent reactivity of high dilutions of extract with group A antisera. It remained in solution during salt addition with only minor amounts recovered in fractions S-1 and S-2. One half of the protein and most of the reactivity with type 14 antisera of the initial extract was found in fraction S-2. A small amount of protein bound rhamnose was also detected in this fraction. It was apparently not present as C polysaccharide, however, since negative ring tests against group A antisera were obtained using concentrations of protein as high as 2 mg/ml (16 μg rhamnose/ml). The rhamnose content of this fraction did not decrease with several reprecipitations from ammonium sulfate. The M protein preparations isolated in this manner have contained from 0.8 to 1.5 per cent bound rhamnose. Attempts to obtain a protein fraction of greater type specific

TABLE 4

Ammonium sulfate fractionation of cell wall extract

Fraction	Source	Protein	Rhamnose	Serologic Reactivity*	
				M protein	C polysaccharide
		mg	*mg*		
	Initial extract	75.5	57.2	++++	++++
S-1	0–0.3 $AmSO_4$ precipitate	7.2	0.3	0	0
S-2	0.3–0.6 $AmSO_4$ precipitate	38.3	0.5	++++	0
S-3	0.6 $AmSO_4$ supernatant	16.3	48.3	+	++++

* Ring test results.

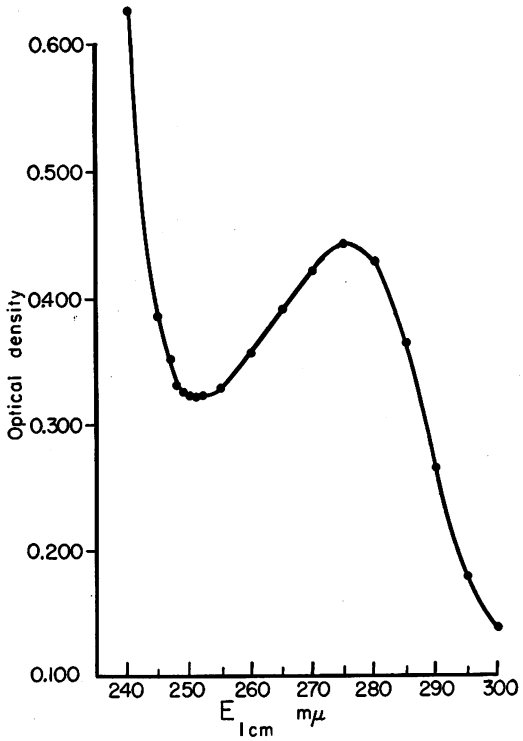

Figure 5. Ultraviolet absorption spectrum of type 14 M protein, 1.0 mg/ml in 0.015 M phosphate buffer, pH 7.5.

serologic reactivity by subjecting fraction S-2 to further fractionation with ammonium sulfate, ethanol, and ion exchange resins have not been successful.

Fraction S-1 was devoid of serologic reactivity. Most of the C polysaccharide of the initial extract was found in fraction S-3. The protein which remained in this fraction showed some reactivity with type specific antisera but this was much less per milligram of protein than was obtained with fraction S-2.

In several experiments the recovery of M protein from cell wall extracts was compared to the amount recovered from extracts of an equal number of whole cells. Differences of 10 per cent or less were found, which would support the view that the cell wall is the principal, if not the only site of localization of the M protein in the streptococcal cell.

Properties of type 14 M protein. The characteristics of type 14 M proteins prepared from cell walls in these studies were similar to those described by Lancefield and Perlmann for type 1 M protein isolated from whole cells. Positive ring tests against anti-M sera were obtained with concentrations of 2.5 to 5.0 μg/ml. Figure 5 shows the ultraviolet absorption spectrum of M protein. It is a typical protein spectrum except for the ratio of 280/260 absorption of 1.37, which is low and denotes a relatively small content of aromatic amino acids.

Several M preparations were examined in a Boskamp microelectrophoresis apparatus and migrated as single symmetrical peaks at pH of 7.4 in 0.05 M phosphate buffer and at pH 8.8 in 0.05 M veronal. Before concluding that these samples consist of a single homogeneous protein, however, more extensive studies would be necessary.

DISCUSSION

The degree of breakage of the group A hemolytic streptococci which has been attained in the Mickle disintegrator is such that the cell walls can be isolated by direct centrifugation without regard to the negligible numbers of the remaining intact cells. The separation of these few whole cells by differential centrifugation was accompanied by large losses of cell walls and became prohibitive when cell wall material was required in relatively large amounts for the preparation of various components. The contribution of the cytoplasmic constituents of the residual intact cells to the total cell wall material was so slight as to escape detection by the analytical procedures employed. The criteria used for the suitability of cell wall preparations in this work rested on determinations of the completeness of cell breakage and of the degree of freedom from cytoplasmic particles using the electron microscope and on the absence of 260 absorbing adherents. The latter is an arbitrary but convenient index, which does not, however, necessarily rule out the presence of other cytoplasmic adherents onto the cell walls.

The difficulties which can be encountered with spectrophotometric measurements on suspended particles was illustrated by the failure to detect the presence of bound nucleic acid in cell wall suspensions. Reliance on this type of data can be misleading unless an attempt is made to eliminate light scattering by suspension in appropriate media (Barer, 1955; De Lamater, 1956), or to correct for it by other methods (Shibata *et al.*, 1954).

The data on the chemical composition of

streptococcal cell walls are in good agreement with the findings of McCarty (1952), Salton (1953) and the extensive survey of Cummins and Harris (1956). The contribution of this component to the dry weight of the streptococcal cell (23 to 25 per cent) is in the range which appears to be characteristic for the cell walls of other gram-positive organisms (Salton, 1956). However, if the type 14 strains are typical for the group A hemolytic streptococci, the portion of the cell wall which is necessary for the preservation of structural integrity amounts to only approx 15 per cent of the whole cells of these organisms. The balance of the cell wall is made up of protein which is neither essential for the maintenance of cell wall structure nor for viability of the organism (Lancefield, 1943), and can be readily removed by the action of trypsin. This protein, however, is essential for type specificity and is therefore identical at least in part with the M antigen.

The relationship of the M protein to the virulence of the group A hemolytic streptococci has gained wide acceptance, although its exact role remains undefined (Lancefield, 1940). Similarly, the importance of type specific antibodies in conferring protective immunity has emerged from studies of experimental infections in animals and from clinical experience (McCarty, 1954). Consequently, the isolation of a purified preparation of M protein which has retained antigenicity may have considerable practical importance in the control of streptococcal infections. To date, however, the prevalent method of recovering the M protein from the streptococcal cell has been the acid-heat extraction of Lancefield. Such a preparation reacts in fairly high dilution with type specific antisera, but is a poor antigen. The data presented in this article may offer a partial explanation for this finding. The M protein is associated with the trypsin sensitive portion which makes up 40 per cent of the cell wall. Two acid-heat extractions remove most of the serologically reactive M protein from cell walls. The total protein of these combined extracts does not exceed 15 per cent of the cell wall weight. It is apparent, therefore, that the extraction procedure solubilizes only a portion of the outer protein surface of the cell wall which, nonetheless, has retained the configuration required for reacting with type specific antibodies. Type specific antigenicity, however, may be a function of a larger protein moiety, a portion of which is still retained on the cell wall surface following extraction.

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SUMMARY

The preparation of cell walls containing 0.1 per cent or less of whole cells from 3 strains of type 14 hemolytic streptococci in the Mickle disintegrator has been described. Several washings of the cell walls in water and phosphate buffer were necessary to eliminate cytoplasmic particles and bound adherents, notably nucleic acids. Cell walls were shown to bind increasing amounts of nucleic acids as the pH was lowered but these were readily removed by washing with phosphate buffer of pH 7.4 or by the action of ribonuclease.

The cell walls were found to make up 23 to 25 per cent of the dry wt of whole cells. Approximately one third of the cell wall is made up of D-rhamnose and hexosamine (probably acetylated) which are the principal constituents of the C polysaccharide. The remaining two thirds is protein in nature, the major portion of which can be removed by the action of trypsin without affecting cell wall structure.

The type specific M protein of streptococci was found to be present principally and probably exclusively on the cell walls from which it could be extracted and partially purified. This protein was destroyed by the action of trypsin. These results suggested a possible explanation for the poor antigenicity of M protein prepared by acid-heat extraction.

REFERENCES

BARER, R. 1955 Spectrophotometry of clarified cell suspensions. Science, **121**, 709–715.

CUMMINS, C. S. AND HARRIS, H. 1956 The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character. J. Gen. Microbiol., **14**, 583–600.

DAWSON, I. M. 1949 *The nature of the bacterial surface*, p. 119. Oxford Univ. Press, London.

DE LAMATER, E. D. 1956 *Bacterial anatomy*, pp. 215–260. Cambridge Univ. Press, Cambridge.

DISCHE, Z. AND SHETTLES, L. B. 1948 A specific

color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem., **175,** 595–603.

ENGLESBERG, E. 1955 Mutation to rhamnose utilization by *Pasteurella pestis*. Bacteriol. Proc., **1955,** 132.

FULLER, A. T. 1938 The formamide method for the extraction of polysaccharides from haemolytic streptococci. Brit. J. Exptl. Pathol., **19,** 130–139.

JERMYN, M. A. AND ISHERWOOD, F. A. 1949 Improved separation of sugars on the paper partition chromatogram. Biochem. J. (London), **44,** 402–407.

LANCEFIELD, R. C. 1928 The antigenic complex of *Streptococcus haemolyticus*. I. Demonstration of a type-specific substance in extracts of *Streptococcus haemolyticus*. J. Exptl. Med., **47,** 91–103.

LANCEFIELD, R. C. 1940 The Harvey Lectures, Series **36,** 251–290.

LANCEFIELD, R. C. 1943 Studies on the antigenic composition of group A hemolytic streptococci. I. Effects of proteolytic enzymes on streptococcal cells. J. Exptl. Med., **78,** 465–476.

LANCEFIELD, R. C. 1947 Technical instructions and procedures employed in the production of streptococcus grouping and typing sera. Brochure of the Hospital of the Rockefeller Institute for Medical Research, New York, New York.

LANCEFIELD, R. C. AND PERLMANN, G. E. 1952 Preparation and properties of type-specific M antigen isolated from group A, type 1 hemolytic streptococcus. J. Exptl. Med., **96,** 71–82.

MAXTED, W. R. 1948 Preparation of streptococcal extracts for Lancefield grouping. Lancet, **2,** 255–256.

MCCARTY, M. 1952 The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. II. Nature of the cellular substrate attacked by the lytic enzymes. J. Exptl. Med., **96,** 569–580.

MCCARTY, M. 1954 *Streptococcal infections*, pp. 130–142. Columbia University Press, New York, N. Y.

NELSON, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., **153,** 375–380.

PALLERONI, N. J. AND DOUDOROFF, M. 1956 Mannose isomerase of *Pseudomonas saccharophila*. J. Biol. Chem., **218,** 535–548.

SALTON, M. R. J. AND HORNE, R. W. 1951 Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. Biochim. et Biophys. Acta, **7,** 177–197.

SALTON, M. R. J. 1953 Studies of the bacterial cell wall. IV. Composition of the cell walls of some gram-positive and gram-negative bacteria. Biochim. et Biophys. Acta, **10,** 512–521.

SALTON, M. R. J. 1956 *Bacterial anatomy*, pp. 81–110. Cambridge Univ. Press, New York, N. Y.

SCHMIDT, W. C. 1952 Group A streptococcus polysaccharide: Studies on its preparation, chemical composition, and cellular localization after intravenous injection into mice. J. Exptl. Med., **95,** 105–117.

SHIBATA, K., BENSON, A. A., AND CALVIN, M. 1954 The absorption spectra of suspensions of living microorganisms. Biochim. et Biophys. Acta, **15,** 461–470.

SUTHERLAND, E. W., CORI, C. F., HAYNES, R., AND OLSEN, N. S. 1949 Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem., **180,** 825–837.