STUDIES ON PREPARATION OF BACTERIAL CELL WALLS AND CRITERIA OF HOMOGENEITY

BOB S. ROBERSON* AND JOHN H. SCHWAB

Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, N.C. (U.S.A.)

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SUMMARY

Group A streptococci can be disrupted in a sonic vibrator in the presence of glass beads to yield large cell wall fragments. A technique is described, involving sucrose zone centrifugation, for collecting cell walls with a high degree of purity, and in good yield, suitable for studies on biological properties. It is concluded that cell walls prepared by the technique of sucrose zone centrifugation display a relatively high degree of homogeneity as indicated by electron microscopy and moving boundary electrophoresis. Immunological studies show the cell wall preparations to be antigenically complex although not necessarily contaminated with non-cell wall antigens.

INTRODUCTION

Recent studies have been reported on the toxic properties of cellular components of group A streptococci[1,2]. Among these components is a factor which produces a chronic, remittent and intermittent multinodular lesion of dermal connective tissue following its intradermal injection into rabbits[1]. This toxic material has been shown to be a complex macromolecule containing the group-specific C polysaccharide[3,4], which is a major constituent of the group A streptococcus cell wall. Subsequent studies have been reported on the relationship between toxic activity and the macromolecular properties of size and configuration of cell wall fragments[5,6]. To pursue these studies further, investigations have been conducted on the efficient preparation of a homogeneous suspension of bacterial cell walls in relatively large quantities.

The method commonly employed for the purification of bacterial cell walls was introduced by DAWSON[7], and extended by SALTON AND HORNE[8]. This technique involves differential centrifugation of disrupted cells and washing sediments with buffers. There have been few modifications of this basic technique, other than treatment with ribonuclease and proteolytic enzymes as employed, for example, by CUMMINS AND HARRIS[9]. Extensive studies on the composition of cell walls derived from group A streptococci have been carried out by MCCARTY[10], and BARKULIS *et al.*[11], using the technique of differential centrifugation.

The present communication describes a modification of the usual technique for preparation of bacterial cell walls, which involves sucrose zone centrifugation. In

addition to electron microscopy, some electrophoretic and immunologic studies on these purified preparations are reported.

METHODS AND RESULTS

Growth of the organisms

A group A, type 1, streptococcus was grown in Todd-Hewitt broth prepared from fresh beef heart[1]. Prior to inoculation, a glucose–phosphate buffer was added to give a glucose concentration of 0.3 %. The medium was inoculated with 4 ml/l of a starter culture in the late logarithmic phase of growth. The culture was incubated for 16–18 h at 35°. Cells were harvested in a Servall S-2 centrifuge, washed twice in cold saline and once with distilled water.

Rupture of the cells and collection of the cell walls

Low percentage rupture of cells by grinding techniques and the high degree of fragmentation and solubilization of cell walls occurring on disruption with sonic vibration, prompted attempts to combine the principles into a single procedure utilizing available equipment. Incorporation of fine glass beads into the vibration mixture was found to produce high percentage rupture. Values obtained for solubilized rhamnose indicated that the mechanism of rupture differs appreciably from sonic vibration alone[12]. The time of vibration was adjusted to obtain maximum rupture with minimum fragmentation. The washed cells from 2 l of culture, suspended in water to a total volume of 10 ml, were mixed with 10 ml Flexolite* No. 18 glass beads. This mixture was vibrated for 25 min at maximum amplitude in a Raytheon 9 kc sonic oscillator. The temperature was maintained at 4° by circulating ice water through the cooling system of the treatment unit.

The vibrated material was transferred to a conical centrifuge tube. Beads and cell material were separated by allowing the beads to settle, withdrawing the supernate, and washing the beads with small aliquots of water which were added to the supernate. This suspension was centrifuged for 30 min at 10,000 rev./min in a Spinco No. 30 rotor, and the supernate was discarded. A rough separation of cell walls and cells was visible in the layered sediment. The more loosely packed cell wall layer was rinsed from the lower cell layer. Two additional sedimentations of both fractions, followed by segregation of the layers, left a pellet composed almost entirely of whole cells. The whole cells thus recovered were combined and utilized again to improve the yield. The suspension from the upper layer of sediments contained mostly cell walls with "granules" and few whole cells.

Sedimentation through sucrose

A sucrose gradient was prepared by using a large bore pipette to layer solutions of decreasing sucrose densities in a 40-ml glass centrifuge bottle. Bottles were layered from the bottom with solutions having the following densities: 1.30 (1.5 ml); 1.25 (4 ml); 1.20 (4 ml); 1.15 (4 ml); 1.10 (4 ml); 1.05 (4 ml); and 1.025 (4 ml). These were placed in the cold for 16 h to allow gradient formation by diffusion.

A uniform dispersion of the cell wall suspension was obtained by placing it in a cellulose centrifuge tube and subjecting it to 30 sec sonic vibration in the cup of the

* Flexolite Mfg. Corp., St. Louis, Mo.

Raytheon oscillator containing 15 ml of water. 3 ml of this suspension was then layered over the sucrose gradient and the bottle tightly stoppered. The bottles were centrifuged at 2,700 rev./min in an International refrigerated centrifuge with a No. 269 rotor, for 1.75 h with controlled acceleration and deceleration according to the method of ANDERSON[13]. To produce uniform acceleration, the auto-transformer speed control was advanced to the setting required to turn the rotor. When the speed reached 100 rev./min, the speed control was advanced 1 unit. After 10 sec, the speed control was advanced an additional unit each 5 sec until the tachometer reached 2,700 rev./min. The speed control was turned back to maintain the speed. Deceleration was accomplished by turning back the speed control 1 unit each 5 sec to a setting which just kept the rotor from stopping.

Three distinct bands were visible and could be removed separately with a pipette. Samples from each band were examined for the presence of whole cells by phase microscopy and Gram's stain. Most of the cells were confined to the lowest band and were discarded. Those bands with no whole cells were combined. The top layer, composed largely of "granules", could be removed at this point. However, since an appreciable quantity of cell wall was present in this layer, segregation of these elements was postponed until after papain treatment, at which time complete separation is more readily achieved. Lower levels of the middle band with rare cells were centrifuged separately. The fractions were washed with water by sedimenting at 17,500 rev./min for 30 min in a Spinco No. 30 rotor. The loose upper layers were again rinsed from the sediments containing the few remaining whole cells and were combined with the cell-free layers. Fractions were resuspended and washed twice with water, and 3 times with pH 7, $I = 0.1$, phosphate buffer by sedimenting at 10,000 rev./min for 20 min in the Spinco No. 30 rotor. The latter washing has been shown to remove ribonucleic acid[14].

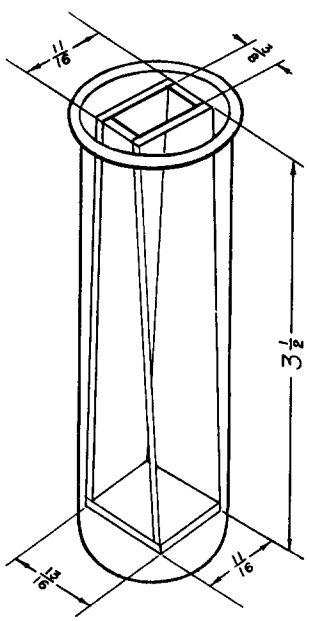

Fig. 1. Sector cell for sucrose zone centrifugation. The rectangular surface lies along the radii of the rotor and perpendicular to the plane of rotation. Dimensions in inches.

The bottles used for the gradient separation in this report, though workable and practicable from the point of view of sterilization and availability, are not ideal. Since the sides of the bottle are parallel, during centrifugation a percentage of particles fanning out from the center of rotation strike the sides, pile up, and slide down the sides. Sharper separation has been achieved by substituting sector cells, constructed to eliminate wall effects. Fig. 1 is a diagrammatic representation of a sector cell constructed for use in sucrose zone centrifugation. The capacity is approx. 17 ml. The cells, constructed from lucite, were held in place in cellulose centrifuge tubes (Lusteroid, IEC, 50 ml), by embedding in a low shrinkage casting plastic*. The cellulose tube and the hardened plastic also provide adequate support for the cell during centrifugation. The body of the cell was constructed from 1/16 inch lucite sheet, the joints fused with acetone. Two rectangular surfaces of the cell were arranged perpendicular to the plane of rotation and lying along radii of the rotor. The other two surfaces were fixed parallel to each other and were trapezoidal. The base dimensions were limited by the internal diameter of the cellulose centrifuge tube.

Papain treatment

Papain was chosen to remove proteins associated with the cell wall because of its wide range of specificity. This enzyme removes 45–50 % of the nitrogen, and is more effective than pepsin, trypsin or chymotrypsin[3]. The cell walls were resuspended in 8.9 ml of water and placed in a small flask. 1 ml pH 7, $I = 0.1$, phosphate buffer was added. 2 mg reduced papain (Nutritional Biochemicals Corporation) in 0.1 ml was added to the suspension, and the pH was adjusted to 7.0. Two drops of chloroform were added, and the flask was incubated with occasional mixing for 5 h at 37°. After incubation, the material was centrifuged for 20 min at 17,500 rev./min (Spinco No. 30 rotor). The sediment was resuspended in water by subjecting it to 30 sec sonic vibration, and sucrose sedimentation was repeated as before. The dense band nearest the top of the gradient, containing many granules was removed and the broad middle band collected. The bands were washed three times with water. The sediment containing the "granules" appeared viscous and transparent. The cell walls appeared opaque and white.

Analysis of fractions: chemical

In order to evaluate the procedure, aliquots for analysis were taken at different stages in the purification process. Rhamnose values, obtained by the method of DISCHE AND SHETTLES[15], were used as an indication of cell wall material in the different fractions, since this sugar is confined to the cell wall structure[10]. Nitrogen values, determined by a micro-Kjeldahl method utilizing Nessler's reagent, were used to calculate rhamnose to nitrogen ratios, which provide an index of relative purity. Table I gives the results of a sample extraction of cell walls from the cells harvested from 5 l of culture medium. The rhamnose of the final preparation indicates that 35 % of the cell walls were recovered by this procedure. The largest loss occurred prior to sucrose gradient separation. Relatively little solubilization of the cell wall occurred during sonic vibration with glass beads, as indicated by the small percentage of rhamnose found in the supernates following centrifugation at 10,000 rev./min for

* Kosto Hobby Crafts Plastic Corp., Washington, D.C. (U.S.A.).

TABLE I

COMPOSITION OF FRACTIONS FROM PURIFICATION PROCEDURE

	Percent of original		*Ratio of Rhamnose*
	Nitrogen	*Rhamnose*	*Nitrogen*
Whole cells	100	100	0.66
Supernates of vibration*			
First vibration	36.9	7.3	0.129
Second vibration**	17.4	3.8	0.143
Total	54.4	11.1	0.134
Cell walls (pre-sucrose)	17.5	64.1	2.22
Cell walls (pre-papain)	13.6	55.3	2.66
"Granules"	2.2	9.9	2.94
Cell walls	4.5	35.1	5.07

* Supernates from centrifugation at 10,000 rev./min, for 30 min.
** From revibration of recovered cells.

30 min (11,500 × *g*). By comparison, a cell preparation sonic vibrated for 1 h without beads requires centrifugation for 1 h at 144,000 × *g* to sediment a comparable percentage of rhamnose. On the other hand, 72 % as much nitrogen is solubilized by sonic vibration with glass beads as that solubilized by sonic vibration alone.

The u.v. absorption spectrum of sonic vibrated cell walls was measured in a Beckman model DU spectrometer. Cell walls which had been treated with papain and washed 3 times with pH 7. phosphate buffer were suspended in water and subjected to sonic vibration without glass beads for 3 h and centrifuged at 10,000 rev./min for 30 min. The resulting supernatant was used for absorption measurements. The curve showed essentially only terminal absorption in the far u.v., with no peak absorption in the region of either 280 mμ or 260 mμ.

Electron microscopy

Fig. 2 shows a composite of electron micrographs representing different phases of purification. Preparations on collodion film were shadowed with chromium at an angle of 15°. Frame 3 shows a mixture of cell walls and "granules". Frames 1 and 2 indicate the high degree of separation achieved between the cell walls and the cellular "granules", and illustrate also the failure of papain to change the gross morphology of these elements.

The occurrence of similar "granules" following disruption of streptococcal cells has been noted by others[8,12]. The nature and origin of these structures is uncertain. SLADE *et al.*[12] report that the mass of the "granules" appears to increase as sonic vibration is continued up to 4 h. Some characteristics of these "granules" can be concluded from the data presented here since, as shown in Fig. 2, the "granule" structures have been separated from nearly all recognizable cell wall material. This preparation has a relatively high rhamnose content, is resistant to papain digestion, and does not show any peak absorption at 260 or 280 mμ. Thus the "granules" appear to be related to the cell wall structure although chemically and morphologically distinguishable. At the present time it seems likely that they represent a complex between certain cell wall fragments and other cell components which aggregate during exposure to sonic vibration.

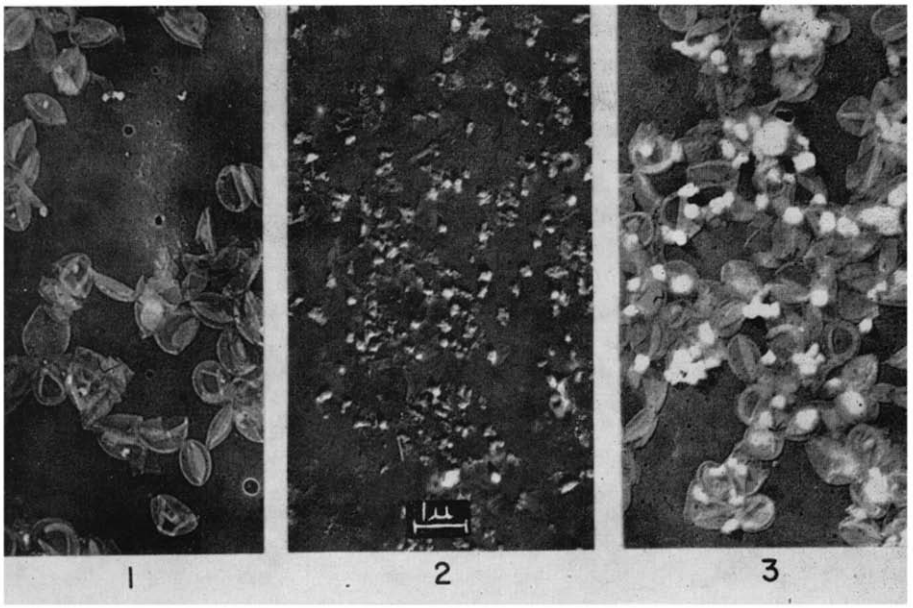

Fig. 2. Composite of electron micrographs showing phases of purification. Shadowed with chromium at an angle of 15°. Frame 1, purified, papain-treated cell wall suspension. Frame 2, "granules" from upper layer of sucrose zone centrifugation. Frame 3, mixture of cell walls and "granules" prior to sucrose zone centrifugation.

No whole cells were found by examining samples of the cell wall preparation by electron microscopy or by phase microscopy. A loopful failed to give evidence of viable cells on blood-agar medium.

Electrophoretic analysis

As a further investigation on the properties of the purified cell wall preparation, electrophoretic analysis was conducted on a suspension dispersed by sonic vibration. Initially, electrophoresis of whole cell walls was attempted. Although there was no evidence of heterogeneity, turbidity was too great for a critical observation of the complete boundary. Therefore, an aliquot of cell wall suspension containing approx. 50 mg dry wt. was suspended in 15 ml veronal buffer, pH 8.6, $I = 0.1$, and sonic vibrated for 2.5 h without glass beads. The suspension was dialyzed for 18 h against buffer, centrifuged for 15 min at 2000 rev./min in an International refrigerated centrifuge (rotor No. 269), and analyzed by moving boundary electrophoresis in a Spinco model H electrophoresis instrument. Fig. 3 shows the descending and ascending patterns obtained at various intervals with a potential gradient of 3.49 V/cm. Considering the complexity of a crude cell extract[12], the pattern shows remarkable homogeneity. There is a faster moving component representing 6 % of the total area, resolvable in both boundaries. Resolution of the principal, skewed boundary into separate components is not observed after 300 min under these conditions of pH, etc. The mobility of the principal peak is $-5.9 \cdot 10^{-5}$ cm^2/V/sec, measuring ∂x to the peak of the boundary. Practical enantiography exists between descending and ascending boundaries throughout the course of current passage. The skewness of the curve could

reflect a polymerization reaction of the type $nB \rightarrow Bn$, the B monomers being produced during vibration. If such a form of modality exists the skewed peak would be considered a single boundary since a homogeneous phase was not produced between the 2 maxima[16]. It is noteworthy that there is enantiographic skewness of the ascending

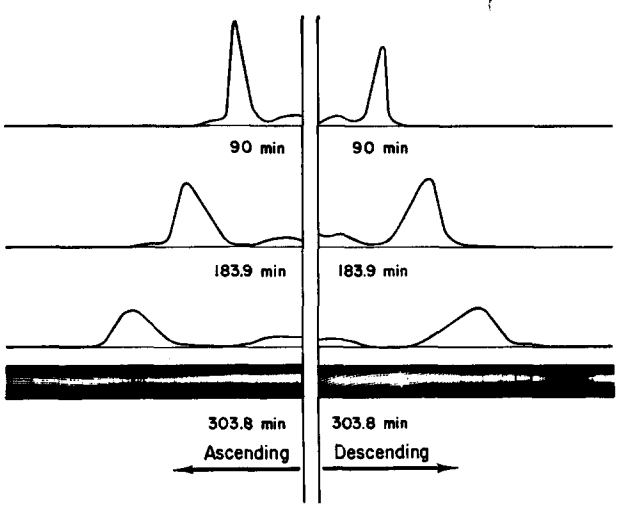

Fig. 3. Moving boundary electrophoretic patterns of cell wall suspension dispersed by 2.5 h sonic vibration. Veronal buffer pH 8.6, I 0.1, at a potential gradient of 3.49 V/cm. The boundary patterns at 90 min were photographed at different diaphragm slit angles.

boundary. This is difficult to explain by either the theory of polymerization and dissociation, or as a bimolecular association[16]. Observations noted during the study of biological activity of vibrated cell walls which suggest a tendency of cell wall fragments to polymerize or to interact include: (a) the rapid disappearance, on standing, of a hemolysin from vibrated cell walls[2]; and (b) the rapid increase in O.D. after initial reduction following treatment of certain cell wall fractions with *Streptomyces albus* enzyme[5].

Immunological studies

Agar diffusion experiments were designed to estimate the immunological complexity of the cell wall preparation. Modifications of both the OAKLEY[17], and the OUCHTERLONY[18] methods were employed. The antisera used were prepared in rabbits against killed streptococcal cell vaccine and against purified streptococcal cell walls[4]. A portion of the antiserum against whole cells was absorbed with a sonic extract of a group A streptococcal variant which produces no group-specific C polysaccharide[19]. This yielded an antiserum which had antibodies reacting only with C polysaccharide[4]. The variant strain, B-455, was kindly supplied by Dr. R. C. LANCEFIELD. These antisera were arranged in agar in positions opposing the sonic vibrated cell wall suspension, and three other streptococcal preparations: the group-specific C polysaccharide obtained from cell walls by FULLER's formamide technique[20]; a sonic extract of whole cells; and a fraction of the whole cell extract (75p60-4), which contained high rhamnose content and an ultraviolet absorption spectrum similar to vibrated cell walls[3]. Table II summarizes the reactions noted with these systems.

The major precipitin band between each antiserum and the vibrated cell wall preparation was common to the band obtained with the isolated C polysaccharide. The presence of at least two additional antigens in the cell wall suspension is established by the reactions between the vibrated cell walls and the antisera against the cell wall preparation and against the whole cells. The presence of another antigen is implied by a fourth precipitin band occurring between the partially purified extract, 75p60-4, and antiserum prepared against the purified cell wall preparation. The absence of this band in reactions with vibrated cell walls suggests that this antigen is present in a quantity too small to produce a visible precipitin reaction in these systems. With all antigen preparations which have been studied the absorbed antiserum shows a single precipitin band, except when reacting with cell wall preparation, as noted in Table II. It is unlikely that a contaminating antigen which was not present in crude cell extracts, would be detectable in the purified cell walls. The possibility is being investigated that the two precipitin lines observed here represent the same antigenic group occurring in fragments with sufficiently different physical properties to produce distinct lines.

TABLE II

PRECIPITIN REACTIONS OF STREPTOCOCCAL FRACTIONS IN AGAR

	Antigen preparations			
	C polysaccharide	*Vibrated cell wall*	*Whole cell extract*	*75p60-4*
Antiserum to whole cells	1 *	3	5	4
Absorbed antiserum **	1	2	1	1
Antiserum to cell wall	1	3	3	4

* Number of precipitin bands developing in agar between antigen and antiserum.

** Antiserum to whole cell vaccine absorbed with a sonic extract of a group A variant producing no group-specific C polysaccharide.

DISCUSSION

The primary purpose of obtaining purified cell wall suspensions was to pursue studies on toxic properties which are derived from the cell wall[5,6]. When applied to disrupted Group A streptococci, methods relying on differential centrifugation alone do not yield a product possessing the purity required for such investigations. These methods were also found to be inadequate for removal of whole cells without large loss of cell walls. Therefore, the modification involving sucrose gradients was introduced. The use of sucrose zone centrifugation serves a dual function. Sucrose, by absorption into the cells increases the apparent density of the cells, thereby increasing the sedimentation differential between them and the cell walls. Compared with conventional centrifugation, the effective distance of sedimentation is also increased, thus increasing and maintaining the separation between elements with different sedimentation rates.

Electron microscopy has often been used to estimate gross particulate impurities in cell wall preparations and the preparations reported here are very homogeneous by this criterion. However, this technique is not satisfactory for studies attempting to assign a biological property specifically to the cell wall structure. Most studies on cell walls have made no attempt to assess the presence of non-particulate contaminating components which, biologically, could be very significant.

Although electrophoretic analysis has been reported on the group specific poly-

saccharides of streptococci prepared by LANCEFIELD technique[21], and on extracts of streptococci solubilized by sonic vibration or shaking with glass beads[22], electrophoresis has not been used to estimate heterogeneity of cell wall preparations. Evidence presented here illustrates the feasibility of reducing the turbidity of the solution by sonic vibration of the cell wall suspension, so that resolution of boundaries by either the cylindrical lens or Rayleigh fringe optical techniques is possible. Dispersion by sonic oscillation apparently does not affect the mobility or electrophoretic heterogeneity of the preparation. However, the degree of polydispersity of the suspension treated in this manner has not been determined.

The immunological analysis of the purified cell walls provides information on the antigenic complexity of the cell wall preparation. Reactions obtained between vibrated cell walls and antisera prepared against the cell wall preparation established the presence of at least two antigenic moities other than the group-specific C polysaccharide. Whether these antigens are an integral part of the cell wall or reflect a degree of contamination with non-cell wall material cannot be ascertained from these experiments. A study comparing protoplasts and protoplast membrane antigens with the cell wall preparations should clarify this question.

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