

# MORPHOLOGICAL, CHEMICAL, AND SEROLOGICAL STUDIES OF THE CELL WALLS OF *RICKETTSIA MOOSERI*

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A membrane surrounding the protoplasm of rickettsiae has been demonstrated electron-microscopically by Plotz *et al.* (1943) and Wissig *et al.* (1956) in air dried preparations, and by Stoker *et al.* (1956) and Tousimis (1956) in ultrathin sections. This limiting membrane may be considered to represent the cell wall of these organisms and to correspond to the analogous structure of bacteria.

Extensive serological and chemical studies have been carried out on isolated bacterial cell walls (see review by Salton, 1956, and Work, 1957). These investigations point a way to establishing a correlation between structure and function which cannot be achieved in studies on intact, whole organisms. Furthermore, characteristic differences in the chemical composition of cell walls of various bacteria have proved of distinct taxonomic value (Cummins and Harris, 1956). Since rickettsiae are considered an intermediate group of organisms between bacteria and viruses, knowledge of the properties of their cell walls are of particular interest.

The present work deals with the preparation and purification of cell walls from *Rickettsia mooseri*, the agent of murine typhus. A new fractionation procedure has been employed, involving the action of sodium desoxycholate on these organisms.

## MATERIALS AND METHODS

*Purified suspensions of R. mooseri.* The rickettsiae were purified from yolk sacs of infected embryonated eggs by the procedure of Wisseman *et al.* (1951). This method yields preparations of viable organisms containing small amounts of contaminating host material as evidenced by the

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complement-fixation test. Such preparations were milky white in appearance and contained approximately 1 mg/ml of rickettsial nitrogen.

*Chemical determinations.* Suspensions of cell walls, prepared as described in Results, were treated overnight at 37 C with 0.1 N NaOH. This treatment partly solubilized the cell walls and gave an opalescent fluid which was used for the various chemical determinations.

Total nitrogen was determined by nesslerization after digestion of the samples with H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub>. Total "protein" was determined by the method of Lowry *et al.* (1951), comparing the developed color with that formed by standard solutions of bovine serum albumin (Fraction V, Armour and Co.). Total phosphorus was determined according to the method of Lowry *et al.* (1954). This procedure was adapted for a range of 0.3 to 3.0 μg phosphorus. Reducing sugar was determined before and after hydrolysis with 2 N HCl carried out for 4 hr in sealed ampoules in a boiling water bath. The procedure employed was that described by Park and Johnson (1949). The results are expressed as glucose equivalents. For the determination of ribose, desoxyribose, and ultraviolet absorption, samples were extracted with 0.5 N perchloric acid at 70 C for 20 min and centrifuged. Ribose and desoxyribose were determined on the supernatant fluid by the methods of Mejbaum (1939) and Ceriotti (1952), respectively. Ultraviolet absorption of such extracts was measured in a Beckman spectrophotometer, model DU. The above chemical determinations were done in triplicate.

Chromatography of the sugar components was carried out on acid hydrolyzates prepared as for the determination of reducing sugar. The hydrolyzates were dried from the frozen state and extracted with anhydrous pyridine. This extract was applied to Whatman no. 1 paper. The solvent systems employed were *n*-butanol:pyridine:water (Jeans *et al.*, 1951); *n*-butanol:acetic acid:

water (Bayly *et al.*, 1951); and ethyl acetate:pyridine:water (Jermyn and Isherwood, 1949). Two or three successive ascending developments were employed. Reducing sugars were detected on the dried paper strips by the ammoniacal silver nitrate method of Trevelyan *et al.* (1950) and the aniline hydrogen phthalate spray of Partridge (1949). Amino sugars were detected with ninhydrin as outlined by Aminoff and Morgan (1948). Glucuronolactone was further delineated by the use of the hydroxylamine-ferric ion spray of Gee and McCready (1957).

Amino acid chromatography was done on samples which were hydrolyzed overnight in 6 N HCl at 105 C in a sealed ampule. Column chromatography was carried out by the procedure of Moore and Stein (1951). Paper chromatography on the same material was done on no. 3 Whatman paper by the method of Slotta and Primosigh (1951) and the color developed by the method of Levy and Chung (1953).

*Electron microscopy.* Cell walls were washed three times in distilled water and treated with 1 per cent osmium tetroxide in isotonic acetate veronal buffer pH 7.35 (Palade, 1952), for periods of 4 to 10 hr. They were then washed three times in distilled water, placed on parlodion-covered<sup>3</sup> grids and shadowcast with uranium at  $\tan^{-1}$  1/3. They were examined in an RCA model E MU-2B electron microscope equipped with a 50  $\mu$  objective aperture. Ultrathin sections of rickettsial cell walls and cells infected with *Rickettsia tsutsugamushi* were prepared by the procedure used by Tousimis (1956).

*Serological procedures.* Complement fixation tests were performed by the method of Plotz *et al.* (1948) employing overnight fixation at 4 C. Murine and epidemic typhus convalescent guinea pig sera were utilized.

Erythrocyte sensitizing substance titrations were performed on aliquots of cell wall preparations and on dialyzed samples of the material released from the rickettsiae. All samples had been previously heated at 100 C for 30 min in the presence of 0.1 N NaOH and then dialyzed against phosphate buffer pH 6.8. The procedure for the hemagglutination test was essentially that described by Chang (1953). A sample of antimurine human convalescent serum was employed as a working standard.

<sup>3</sup> 1 Per cent Parlodion (Pyroxylin Purified, Mallinckrodt) in amyl acetate.

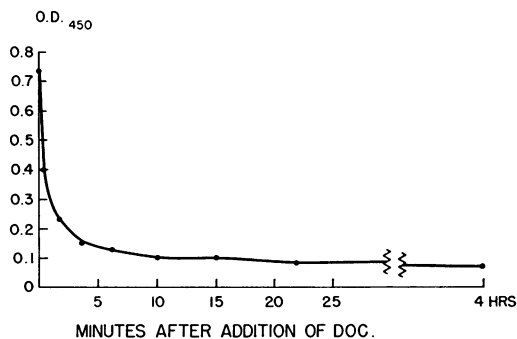


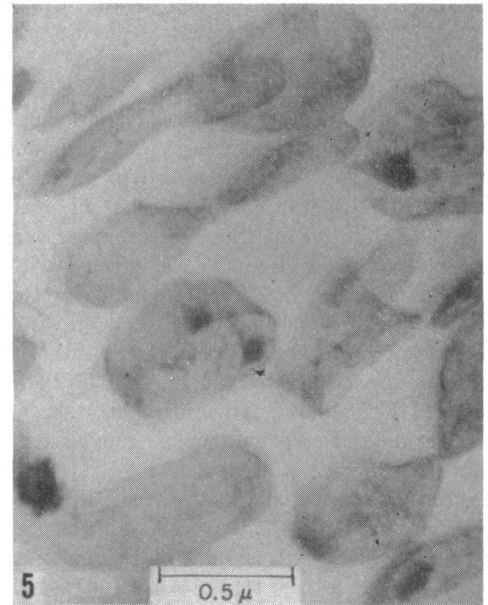
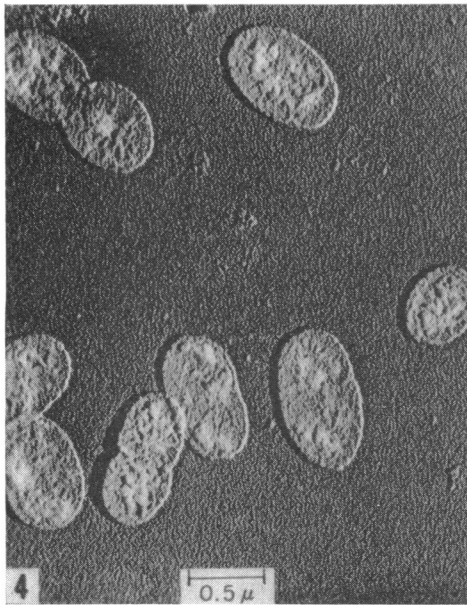
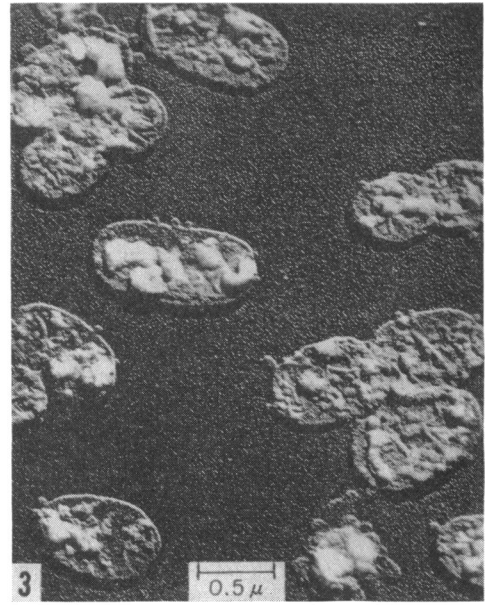
Figure 1. The effect of 1% sodium desoxycholate on the optical density of a suspension of *Rickettsia mooseri*. Samples were taken over a 4 hr interval. The treatment was carried out at 45 C.

## RESULTS

*Preparation of purified cell walls of R. mooseri.* The addition of sodium desoxycholate (Difco) to purified suspensions of *R. mooseri* resulted in a rapid decrease in turbidity<sup>4</sup> (figure 1). Electron-microscopic examination of this material following high speed centrifugation revealed that the sediment consisted of cell walls containing various amounts of cytoplasmic material. In order to obtain cell walls of maximum morphological purity, the following conditions of extraction were studied: time, temperature, desoxycholate concentration and pH. The conditions which gave the best preparations of cell walls free of cytoplasmic material, were as follows: Rickettsial suspensions were frozen and thawed twice, diluted 1:10 or 1:20 with an aqueous solution of desoxycholate at pH 7.0 (1 per cent final concentration), and incubated at 45 C under constant agitation for 4 hr.

Treatment with lower concentrations of desoxycholate, shorter time periods, lower temperatures or different pH, resulted in incompletely extracted cell walls containing appreciable amounts of cytoplasmic material. On the other hand, higher temperatures (55 to 70 C), although very efficient in removing cytoplasmic material, broke down a portion of the cell walls into small, granular fragments. Under the conditions described, the optical density of the final preparation was 10 to 15 per cent that of a similar dilution of untreated rickettsiae.

<sup>4</sup> This phenomenon brings to mind the "flash" lysis of pneumococci by bile salts and by some synthetic detergents.



*Figure 2.* Air dried, shadowed, preparation of *Rickettsia mooseri* showing the cell walls surrounding the central, shrunken protoplasm.

*Figure 3.* Sample taken 10 min after the addition of 1% sodium desoxycholate to a suspension of *Rickettsia mooseri*. Residual masses of cytoplasmic material are seen. The globular structures protruding from the surface of the cell walls may represent the extrusion of cytoplasmic material.

*Figure 4.* Purified preparation of cell walls. Sample taken 4 hr after the addition of 1% sodium desoxycholate. The cell walls are wider than the untreated rickettsiae owing to their flattening on the supporting film.

*Figure 5.* Randomly oriented section of purified cell walls. Circular ribbons and flat cuplike structures are seen, indicating that the cell walls had preserved a three dimensional appearance throughout the embedding procedure.

The cell walls were purified by the following procedure: the suspensions containing desoxycholate were centrifuged at  $24,000 \times G$  for 30 min in a Spinco Model L preparative ultracentrifuge (Rotor no. 30). The supernatant was decanted and the sediment resuspended in distilled water and washed three times. The pellet containing the sedimented cell walls was translucent and easily resuspended.

*Morphologic observations of cell walls.* Under the electron microscope, air dried, shadowed preparations of purified cell walls appeared as flat, ovoid structures whose surface did not show any detectable evidence of rupture. The small masses of electron opaque material which were seen, probably consisted in part of unextracted cytoplasmic residues. This material was not present to the same extent in different preparations and was not affected by repeated washing of the cell walls in 0.5 or 1 N NaCl. In air dried preparations of intact untreated rickettsiae, stained for 1 hr with 1 per cent  $OsO_4$ , the cell walls were seen at the periphery of the organisms, surrounding a dense, shrunken cytoplasm (figure 2). Samples taken at different times during the desoxycholate treatment revealed that the irregularly distributed, amorphous masses of cytoplasmic material progressively disappeared from within the cell walls. Figure 3 represents a sample taken 10 min after the addition of desoxycholate. Small, globular elements which were seen attached to the surface of the cell walls may represent the extrusion of cytoplasmic components. These globules were apparently solubilized by further treatment

with desoxycholate since samples taken after exhaustive extraction did not show the presence of similar material.

Suspensions of purified cell walls were stained with  $OsO_4$ , embedded in methacrylate and sectioned for examination in the electron microscope. Figure 5 shows a relatively thick section (estimated thickness of  $0.15 \mu$ ) composed of segments of cell walls which preserved their three-dimensional shape. Circular ribbons, and cuplike longitudinal sections can be seen, as expected from the random orientation of the cell walls in the embedding medium. Ultrastructural details cannot be interpreted in this material.

The morphology of cell walls in whole organisms was examined in thin sections of tissue culture cells (rat fibroblast, strain 14pf) infected with *R. tsutsugamushi* (figure 6). There are two clearly defined limiting membranes having a total thickness of 180 to 300 A, while a less dense portion between the two membranes varies in thickness from 80 to 120 A. The individual membranes range from 45 to 70 A each.

*Chemical constituents of cell walls of R. mooseri.* Only preparations in which minimal amounts of cytoplasmic material were seen within the cell walls (figure 4) were used for analytical work. Owing to the small amounts of material available, chemical determinations were restricted to a few substances. The results of chemical determinations are presented in table 1. The values are given in terms of the total nitrogen content. No visible color was obtained with the Ceriotti reagent for desoxyribonucleic acid (DNA), although

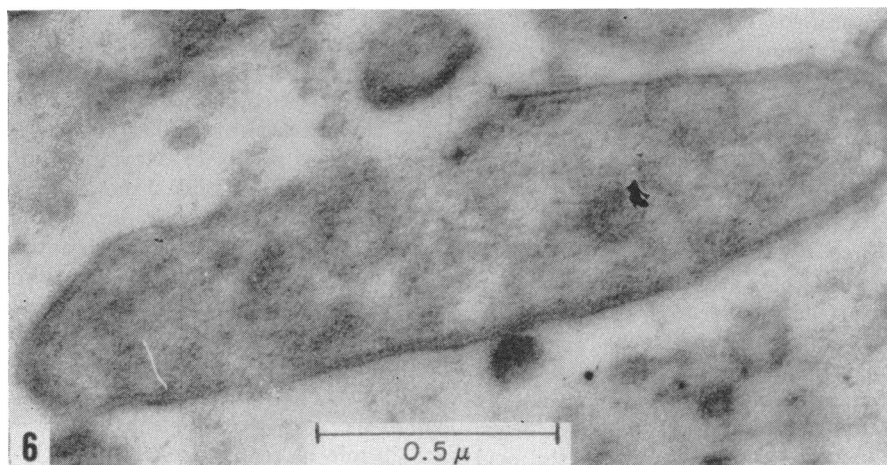


Figure 6. Section of *Rickettsia tsutsugamushi* in a rat fibroblast cell (strain 14pf). Two limiting membranes surround the organisms except for the torn upper portion.

TABLE 1  
Chemical constituents of cell walls of  
*Rickettsia mooseri*

Constituent	Lot Number			
	M34-2	M36-2	M40-3	M40-4
<i>mg/100 mg N</i>				
"Protein" (Lowry-Folin).....	634	752	679	619
Reducing sugar				
Pre-hydrolysis.....	13.7	22.2	26.1	15.2
Post-hydrolysis.....	81.8	94.0	98.3	52.9
Ribose.....	3.0	7.7	2.8	3.1
Phosphorus.....	11.5	—	20.3	5.6
Optical density at 265 $\mu$ in OD units/100 mg N.....	1.75	0.92	0.44	1.13

ten times the usual quantity of cell wall preparation was employed. Easily discernible reactions could be obtained with as little as 2.0  $\mu$ g of a highly polymerized DNA standard (Worthington).

Attempts were made to extract the ribose and the ultraviolet absorbing material from cell walls. For this purpose, suspensions were treated with 1.0, 0.5, and 0.2 N NaCl at room temperature for 2 hr. Between 40 and 50 per cent of the total ribose and ultraviolet absorbing material was removed by this treatment. Incubation with 100  $\mu$ /ml of ribonuclease (Worthington) in M/15 phosphate buffer at pH 7.5 for periods up to 2 hr at 37 C resulted in a 60 per cent decrease in the ribose content of the cell walls. Electron microscopic examination of ribonuclease and salt treated preparations of cell walls revealed no morphological changes resulting from this treatment.

The amino acid composition of the cell walls was studied on acid hydrolyzates by the method of Moore and Stein (1951). The results are given in figure 7. Neutral and dicarboxylic acids, small amounts of tyrosine and phenylalanine and only one basic amino acid, lysine, were found. These amino acids were also identified on paper chromatograms of the same hydrolyzates. In addition, traces of proline and methionine were found on the paper chromatograms. Special tests for cysteine or tryptophan were not performed.

The sugar composition of cell walls was determined by paper chromatography of the acid hydrolyzates. Tentative identification of the four components was made by comparison of  $R_f$

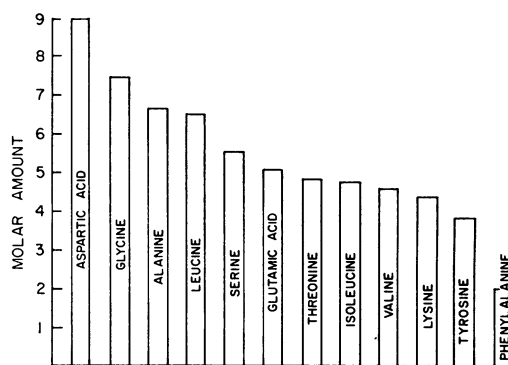


Figure 7. Proportional amounts of amino acids found in an acid hydrolyzate of cell walls by column chromatography. All these amino acids were, in addition, qualitatively identified by paper chromatography. No special tests were performed for cysteine or for tryptophan.

values to standard sugar solutions in three different solvent systems. The spot identified as hexosamine, in addition to reacting with reducing sugar sprays, gave a positive ninhydrin reaction. No other reducing sugar, ninhydrin-reacting material was demonstrated. Glucuronic acid gave double spots in the systems employed. The component with the highest  $R_f$  was identified as glucuronolactone on the basis of its positive reaction with the hydroxylamine-ferric ion reagent. A semiquantitative estimate of the concentrations present in the hydrolyzates, as determined by the intensity of the reducing sugar spots, showed the following: glucose > hexosamine > glucuronic acid > galactose.

*Serological properties of cell walls.* Rickettsial antigens which fixed complement with the sera of immune guinea pigs were destroyed by 4 hr desoxycholate treatment at 45 C. Samples taken after 2½ hr treatment at 40 C possessed antigenic activity. The results of a typical complement fixation test given in table 2 showed that the cell walls contain a significant amount of group reactive antigen. The bulk of such antigen, however, was released into the medium through the extraction of the cytoplasm by desoxycholate. The fate of the type specific antigen originally present in the intact rickettsiae could not be ascertained. Attempts were made to remove the group specific activity by shaking the cell walls for 30 min at room temperature with 0.2, 0.5, and 1.0 N NaCl or for 2 hr with ribonuclease and desoxyribonuclease. None of these treatments

TABLE 2  
Complement fixation by cell walls of  
*Rickettsia mooseri*

Sample	Antisera*	
	Murine typhus	Epidemic typhus
Intact <i>R. mooseri</i> .....	480	60
Cell walls.....	240	240
Dialyzed supernatant of cell wall preparation.....	3,840	1,920

\* Reciprocal of titer.

TABLE 3  
The localization of erythrocyte sensitizing substance  
in the cell walls of *Rickettsia mooseri*

Preparation*	Cell Walls	Dialyzed Supernatant of Cell Wall Preparation
A	1,280†	16
B	256	<8

\* Preparation A contained approximately three times more rickettsial nitrogen than B.

† Reciprocal of the titer completely sensitizing 0.1 ml of thrice washed, packed human O erythrocytes.

resulted in a significant decrease of the group specific titer.

It is apparent from table 3 that a high concentration of erythrocyte sensitizing substance is present in the cell walls of *R. mooseri*. In preparation A the erythrocyte sensitizing substance titer of cell walls was 80-fold greater than that in the lysate, and in preparation B there was at least a 40-fold difference.

Although the untreated rickettsial suspensions gave a low titer when tested with normal anti-yolk sac antiserum, the purified cell walls were uniformly negative. This indicates that the small amount of contaminating host material was significantly reduced during the preparation of the purified cell walls.

#### DISCUSSION

The procedure for purification of cell walls of *R. mooseri* developed in the current investigation has provided material which is suitable for chemical and serological determinations. The isolated cell walls were apparently only slightly degraded and were morphologically comparable

to the homologous structures of air dried or sectioned rickettsiae. However, the degree of purity, in terms of the absence of cytoplasmic material in the cell walls, has not been determined critically. The two types of findings which in this light acquire greatest significance are the presence of major components and the absence of others in appreciable amounts. On the other hand, substances present in small concentrations varying between different lots may represent contamination. In the particular case of ribonucleic acid this is further emphasized by the maintenance of morphological integrity after partial removal of this substance. Material of greater purity might also be obtained by using other methods of fractionation which, moreover may give information about the possible removal of certain substances by the desoxycholate extraction. The surface active nature of desoxycholate may play a role, for instance, in the extraction of lipid constituents.

The serological analysis of the cell walls of *R. mooseri* demonstrated the presence of a group specific complement fixing antigen, which, however, is not primarily localized in these structures. Indeed, this activity may be manifested by the cell walls only by virtue of contamination by cytoplasmic material. Attempts to detect a type specific antigen in cell walls were unsuccessful, although it is present in the intact organisms before desoxycholate treatment. The lack of demonstrable type specific antigen in cell walls could be the result of a number of factors, including the destruction of such material by desoxycholate or masking by group reactive antigen. The high erythrocyte sensitizing substance activity of cell walls and the low titers found in the extracted rickettsial protoplasm indicate that this antigen is localized in the cell wall of the organism.

The chemical determinations indicate that the two major constituents of the rickettsial cell wall are made of amino acids and oligosaccharides. In this respect, their cell walls resemble those of bacteria (Salton, 1953). Amino acid analyses of hydrolyzates suggested that the cell walls may not contain ordinary protein since only 12 amino acids were identified and at least 3, methionine, arginine, and histidine, were not present in appreciable amounts. On the basis of these findings it cannot be stated whether rickettsial cell walls resemble those of gram-positive

bacteria which contain as few as 4 amino acids, or those of gram-negative bacteria which possess 16 or more (Salton, 1956; Cummins and Harris, 1956). Diaminopimelic acid, which has been found in bacterial cell walls, was not identified in those of rickettsiae. Glucuronic acid, which was identified chromatographically in the present study, has not been reported to be a component of the polysaccharide moiety of bacterial cell walls (Salton, 1956).

Sodium desoxycholate has been employed by Crocker (1956) for the purification of meningopneumonitis virus. Electron microscopic examination of preparations treated with this substance revealed the presence of empty sacs which, in the light of the present investigation, may represent cell walls. It would be of considerable taxonomic interest to compare the composition of such structures in some of the large viruses with that of the cell walls or rickettsiae and bacteria. The present findings suggest certain structural similarities between the cell walls of rickettsiae and those of bacteria which may help elucidate the relationship between these organisms.

#### SUMMARY

Cell walls of relative morphological purity were prepared from *Rickettsia mooseri* by controlled treatment with sodium desoxycholate. Chemical analyses revealed that these structures contain amino acids, polysaccharide, and small amounts of ribonucleic acid. Twelve amino acids were identified in acid hydrolyzates. No appreciable amounts of methionine, arginine, and histidine were found. The polysaccharide moiety contains glucose, hexosamine, glucuronic acid, and galactose, as determined by paper chromatography.

Complement fixation tests demonstrated the presence of a group reactive antigen. An erythrocyte sensitizing substance was found in much greater amounts in the cell wall than in the rest of the organism.

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