The Cell Wall of *Rickettsia mooseri*

I. Morphology and Chemical Composition[1]

WILLIAM H. WOOD, JR.,[2] AND CHARLES L. WISSEMAN, JR.

Department of Microbiology, University of Maryland School of Medicine, Baltimore, Maryland

Cell walls prepared by mechanically disrupting intact *Rickettsia mooseri* (*R. typhi*) were examined in an electron microscope and analyzed chemically. Electron micrographs of metal-shadowed and negatively stained rickettsial cell walls revealed no significant differences, except for smaller size, from bacterial cell walls prepared in a similar manner. The chemical composition was complex, and resembled that of gram-negative bacterial cell walls more closely than that of gram-positive bacterial cell walls. *R. mooseri* cell walls contained the sugars, glucose, galactose, and glucuronic acid, the amino sugars, glucosamine, and muramic acid, and at least 15 amino acids. Diaminopimelic acid, a compound hitherto found only in bacteria and blue-green algae, was demonstrated in rickettsiae for the first time. Teichoic acids were not detected. The compounds identified accounted for about 70% of the dry weight of the cell walls.

Rickettsiae possess many characteristics in common with bacteria. Information accumulated in the past few decades suggests that rickettsiae are, in fact, more closely related to bacteria than to viruses (14, 21, 29), even though they do not grow in an acellular environment under known natural circumstances. [The taxonomic position of "*R.*" *quintana* (43), the Canadian vole agent (6) and comparable organisms which can now be grown on artificial cell-free media is not clear at this time. Moreover, some bacteria, e.g., *Mycobacterium leprae*, have not been grown on artificial media. Nevertheless, the pathogenic rickettsiae of man, exclusive of the organism causing trench fever, still appear to be obligate intracellular parasites in both mammalian and arthropod hosts.]

These organisms possess a rigid outer membrane which is resistant to enzymatic treatment with trypsin, deoxyribonuclease, and ribonuclease (37) and which appears similar to bacterial cell walls when viewed in an electron microscope (2, 3, 37). Moreover, this cell wall structure contains several amino acids and the amino sugar,

muramic acid, a compound common to all bacteria (1, 29, 37).

Techniques useful for preparation of bacterial cell walls have also yielded a stable cell wall fraction of *Rickettsia mooseri* (*R. typhi*) suitable for extending the study of its fine structure and chemical composition.

MATERIALS AND METHODS

Preparation of rickettsiae and cell walls. The Wilmington strain of *R. mooseri* (25), used throughout these studies, was in the 39th egg passage at the University of Maryland, but had an uncertain history, involving several guinea pig and egg passages at Harvard University and the Walter Reed Army Institute of Research prior to its receipt in Baltimore in 1954. Infected yolk sac pools, which had been stored at −70 C, were partially purified by differential centrifugation (44), further separated from yolk sac material on a linear sucrose density gradient (31), and washed three times in distilled water to yield the preparation designated as *purified whole rickettsiae.*

To prepare cell walls in maximal yield, 9 ml of purified whole rickettsial suspension (optical density, 7.0 to 9.0 at 450 mμ, Bausch & Lomb Spectronic-20 colorimeter, 0.5-inch cuvettes) combined with 5 g of Ballotini beads (no. 12, 0.279 to 0.381 mm in diameter) was vibrated at maximal amplitude for 18 to 22 min at 4 C in a Mickle Tissue Disintegrator (36). The cell wall material was filtered through coarse sintered glass. The retained glass beads were washed twice with distilled water. The filtrate of the original fluid and the washings were centrifuged separately at 34,880 × *g* for 20 min (no. 30 rotor, model L Spinco

ultracentrifuge). The supernatant fraction of the original fluid was saved and was labeled *cytoplasm*; the wash fluid supernatant fractions were discarded. The combined sediments were centrifuged through a 10 to 40% linear sucrose density gradient, yielding two bands: a dense upper layer containing the cell walls and a faint lower one containing intact whole rickettsiae. The upper band (*cell walls*) was collected and washed twice with distilled water; a sample was examined by electron microscopy and lyophilized.

Electron microscopy. Aqueous suspensions of whole rickettsiae and cell walls were prepared for electron microscopy by placing a drop of the test material upon a Formvar-coated 200-mesh copper grid; after 2 to 3 min for settling, the drop was removed with filter-paper strips. When dry, grids were stained for 4 hr with osmium vapors (0.14 M acetate Veronal buffer, pH 7.6, containing 1% osmium tetroxide). The grids were shadowed with 40 mg of chromium in a Kinney vacuum evaporator (angle of 53°). With other stains, aqueous suspensions of the test material combined with the specific stain [0.5% (w/v) uranyl acetate, etc.] were sprayed onto Formvar-coated grids. The samples were examined in an RCA EMU 3 C electron microscope.

Basic chemical analyses. Total nitrogen was estimated by digestion with sulfuric acid and hydrogen peroxide, followed by nesslerization; total phosphorus, by digestion with sulfuric acid and hydrogen peroxide followed by a modified Fiske-SubbaRow color development of the phosphomolybdate ion (13); and total protein, by the Lowry method with the use of the Folin-Ciocalteau reagent (23). Reducing sugars were determined after hydrolysis with 2 N HCl for 2 hr at 105 C by the method of Park and Johnson (28).

Total nucleic acid in 0.5 N perchloric acid extracts (70 C, 20 min) was calculated from the absorbancy at 265 mμ (Beckman DU spectrophotometer) and from the phosphorus content by use of data from the nucleic acids of *Escherichia coli* (43).

Total hexosamine was determined by the Elson-Morgan reaction (glucosamine-HCl standard; range, 4 to 40 μg) (34). For analysis, a hydrolysate (4 N HCl for 4 hr at 105 C) was dried in vacuo, resuspended in a small amount of water, and applied to a column (12 by 40 mm) of Dowex 50-W x 4 (200 to 400 mesh, H+ form). The eluate obtained with 20 ml of 0.5 N HCl was dried in vacuo and redissolved in water for amino sugar determination.

Amino sugar analysis. For either paper or column chromatography, cell wall material was hydrolyzed with 4 N HCl for 4 hr at 105 C, dried in vacuo over NaOH pellets and suspended with water several times, and finally filtered to remove humin. No corrections were made in calculations for release and degradation of amino sugars during hydrolysis.

Single-dimension ascending chromatography on Whatman 1 and 3 MM papers utilized ethyl acetate-pyridine-acetic acid-water, 5:5:1:3, and *n*-butanol-pyridine-water, 8:8:4 (23) as solvents. Standards were glucosamine-HCl and galactosamine-HCl. Ninhydrin in acetone (2%, w/v) and aniline hydrogen phthalate (11) were used to locate compounds.

In column chromatography, small amounts of hydrolysate were applied to columns (0.8 by 60 cm) of Dowex 50-W x 4 (200 to 400 mesh). Fractions (1 ml) were collected (Gilson Medical Electronics Fraction Collector) on elution with 0.3 N HCl (15), neutralized with 2 N NaOH, and analyzed in the Elson-Morgan reaction outlined in the previous section. Readings were made between 490 and 570 mμ at 30 min and 18 hr, as suggested by Krause and McCarty (22). The 505/530 mμ ratio at 18 hr identified the components of the chromatographic peaks, since glucosamine gives a ratio of 0.8 to 1.0; muramic acid, a ratio of 2.5 to 3.1; and galactosamine, a ratio of 0.6 to 0.71. HCl (6 N) hydrolysates of 5% trichloroacetic acid precipitates of *Micrococcus lysodeikticus* were employed as column and paper chromatography standards for the amino sugars and muramic acid.

Amino acids analysis. Cell wall material was hydrolyzed with 6 N HCl for 18 hr at 105 C and was treated as described for the amino sugar hydrolysates. Two-dimensional chromatography on Whatman no. 1 paper was used, with phenol-water, 80:20 (w/v) plus either butanol-acetic acid-water, 120:30:50 (7) or ethyl acetate-pyridine-acetic acid-water, 5:5:1:3, as solvent systems. The location reagent was ninhydrin in acetone (2%, w/v). Column chromatography was performed with a Beckman Amino Acid Analyzer (model 122). The L forms of the amino acids were used as standards.

Microbiological assay of diaminopimelic acid (DAP), by the method of Moulder et al. (26), utilized the M-145 auxotropic strain of *E. coli*, kindly furnished by James Moulder. Strain M-145 was maintained on Penassay Agar plus 5 mg of L-methionine, 25 mg of L-threonine, L-lysine, and L-leucine, and 20 mg of DAP per liter.

Sugar analysis. Reducing sugars were identified chromatographically in 2 N HCl hydrolysates of cell wall material treated for 2 hr at 105 C and handled as described for the amino sugar hydrolysates. Ascending chromatograms on Whatman no. 1 and 3 MM paper were developed with ethyl acetate-pyridine-acetic acid-water (5:5:1:3) and butanol-acetic acid-water (12:3:5), and were sprayed with ammoniacal silver nitrate or aniline hydrogen phthalate (41).

RESULTS

Morphology. Morphological study of rickettsiae and their fractions by electron microscopy regularly preceded observations on chemical composition and biological properties in all instances. The chromium-shadowed rickettsiae purified in a sucrose density gradient, the starting material for cell wall production, resembled closely other rickettsiae described recently (2, 37, 44). Treatment of rickettsiae in a Mickle Tissue Disintegrator resulted in a product (Fig. 1), considered to be the cell wall, which was similar to that described for other members of the family *Rickettsiaceae* and for bacteria (2, 35). Conditions

FIG. 1. *Rickettsia mooseri cell wall, fixed in osmic acid and shadowed with chromium.* × *62,100.*

for maximal cell wall yield were constant from experiment to experiment, and resulted in structures whose morphology did not vary greatly.

Various staining techniques (lead acetate, lead hydroxide, phosphotungstic acid, uranyl acetate), used in an attempt to study the fine structure of *R. mooseri* and its cell wall, achieved little increase in detail in comparison with chromium-shadowed specimens. Negative staining with phosphotungstic acid failed to reveal any structure resembling a capsule. In each of two experiments, samples of a suspension of whole rickettsiae and the derived cell walls were weighed and compared in an attempt to estimate the proportion of whole rickettsiae formed by the cell wall. Since quantitative yield of cell walls was considered unlikely, the estimated per cent weights were probably low. Thus, electron micrographs revealed an 85 to 90% conversion to cell wall, giving an error of approximately 10 to 15%. The results (Table 1) show cell wall percentage weights of 18.8 and 23.4, respectively, a figure within the general range of weights reported for bacterial cell walls (32, 38) and close to the calculated cell wall space of the same organism in the plasmolysis experiments of P. J. Provost,

W. F. Myers, and C. L. Wisseman, Jr. (Bacteriol. Proc., p. 133, 1963). Because quantitative recovery of cytoplasmic material by lyophilization was not accomplished, "total" weight relationships of the rickettsial fraction could not be estimated as had been done by Ribi (32) for *Salmonella enteritidis.*

Chemical composition. The basic chemical constituents of murine typhus rickettsiae and their derived cell walls are presented in Table 2. The protein and nitrogen contents did not differ markedly; however, reducing sugar and hexosamine levels were greater, and nucleic acid content was significantly less, in the cell wall fraction than in intact rickettsiae. Because there was proportionately less reduction of phosphorus than of nucleic acid, other phosphorus-containing compounds were suspected. No lipid analyses were undertaken. Three attempts to demonstrate the presence of teichoic acids in the cell walls of *R. mooseri* were unsuccessful. In these experiments, comparison of phosphorus content of cell walls before and after 18-hr extraction with 10% trichloroacetic acid at 4 C disclosed no significant

TABLE 1. *Weight relationship of Rickettsia mooseri cell walls to whole rickettsiae*

Description of material	Dry wt (μg/ml)	
	Expt 1	Expt 2
Whole rickettsiae...........	1,310	770
Cell walls.................	249	180
Per cent of whole rickettsial weight contributed by cell walls....................	18.8%	23.4%

TABLE 2. *Chemical composition of Rickettsia mooseri and its cell wall*

Constituent	Whole rickettsiae (%, dry wt)	Cell walls (%, dry wt)
Nitrogen..................	11.5 (2)[a]	9.0 (3)
Protein...................	56.0 (2)	60.3 (3)
Reducing sugar[b]...........	6.2 (2)	9.5 (3)
Hexosamine[c]..............	0.8 (2)	2.8 (3)
Total nucleic acid[d]........	5.5 (2)	0.7 (2)
Phosphorus...............	1.8 (2)	1.0 (3)

[a] Number of determinations.

[b] Determined after hydrolysis with 2 N HCl for 2 hr at 105 C.

[c] Determined after hydrolysis with 4 N HCl for 4 hr at 105 C.

[d] Determined on 0.5 N perchloric acid extracts for 20 min at 70 C.

reduction, suggesting an absence of teichoic acids (5). Moreover, no ribitol was detected on paper chromatograms of HCl hydrolysates of cell walls, with alkaline silver nitrate indicator.

The basic classes of compounds identified account for 60 to 70% of the cell wall dry weight. Quantitative analyses of amino acids and amino sugars of the cell walls account for roughly the same amount. About 20 to 30% remains unidentified and, by inference from prior chemical analyses of rickettsiae (8–10, 33, 37, 39, 40), much of this might be lipid.

Amino sugars. One-dimensional paper chromatography of cell wall hydrolysates demonstrated two spots, each of which reacted with both ninhydrin and aniline hydrogen phthalate, a reaction common to amino sugars. The first coincided with glucosamine standards and the second with an amino sugar, derived from acid hydrolysates of *M. lysodeikticus*, with the migratory characteristics of muramic acid (11). Upon elution of cell wall hydrolysate from a Dowex 50 x 8 ion-exchange column, two fraction peaks were detected with the Elson-Morgan reagent, as shown in Fig. 2. By determining the 18-hr 505 $m\mu$/530 $m\mu$ ratios, the two peaks gave readings typical of glucosamine (0.81) and muramic acid (1.76). Similar curves were obtained with the acid hydrolysates of *M. lysodeikticus*. The materials from the peaks, when concentrated and analyzed by one-dimensional paper chromatography, gave results identical to those found with the crude hydrolysate.

Sugars. Rickettsial cell wall hydrolysates, analyzed qualitatively for sugars by paper chromatography, revealed the presence of glucose,
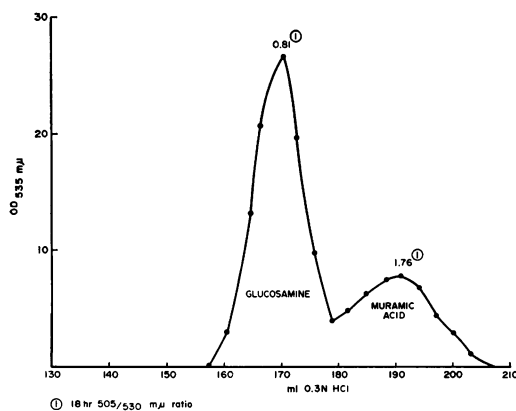
galactose, and glucuronic acid. Schaechter et al. (37) reported the same sugars in *R. mooseri* cell walls prepared by lysis with sodium deoxycholate.

Amino acids. Amino acids were present in rickettsial cell walls in great variety, as shown in Table 3. Of special interest is the fact that DAP, not reported previously in rickettsiae, was detected and measured by three different techniques. Thus, a faint, slowly migrating spot which coincided with the DAP standard was detected with ninhydrin reagent on two-dimensional paper chromatograms of hydrolysates. Elution of a peak from the Moore-Stein column which coincided with a DAP standard provided further proof. Finally, both whole cell and cell wall hydrolysates supported the growth of the M-145 DAP-requiring strain of *E. coli*. The latter two methods yielded comparable quantitative results

TABLE 3. *Relative amounts of amino acids in Rickettsia mooseri cell walls*[a]

Compound	Moles/g (dry wt) $\times 10^{-4}$	Per cent dry wt
Aspartic acid	0.357	4.8
Threonine	0.498	5.9
Serine	0.259	2.7
Proline	0.107	1.2
Glutamic acid	0.335	4.9
Glycine	0.347	2.6
Alanine	0.350	3.1
Valine	0.057	0.7
Diaminopimelic acid	0.025	0.5
Isoleucine	0.205	2.7
Leucine	0.297	3.9
Tyrosine	0.090	1.6
Phenylalanine	0.155	2.6
Glucosamine	0.068	1.2
Muramic acid	0.112	2.7
Lysine	0.371	5.4
Ammonia	1.216	20.7
Arginine	0.128	2.2
Total	4.977	69.4

[a] Based upon determinations in an Amino Acid Analyzer.

FIG. 2. *Determination of the amino sugars in Rickettsia mooseri cell wall hydrolysates by chromatography on 0.8 × 50 cm Dowex 50-W × 4 column (200 to 400 mesh). Elution with 0.3 N HCl. Flow rate, 8 to 12 ml/hr.*

TABLE 4. *Diaminopimelic acid content of whole organisms and cell walls of Rickettsia mooseri*

Material	Prepn designation	Diaminopimelic acid content (μmoles/mg)	
		Chromatographic assay	Microbiological assay
Whole cells	A	—	0.007
Cell walls	A	0.025	0.033
Cell walls	C_1	—	0.017

(Table 4), and the ratio of whole cell to cell wall DAP, as determined by the microbiological assay method, is comparable to the proportion of whole dry cell weight contributed by the cell wall, suggesting that the measured component was largely, if not completely, confined to the cell wall.

DISCUSSION

Relatively pure suspensions of intact typhus rickettsiae and their cell walls were obtained with purification and disintegration techniques found successful with *Coxiella burneti* and many bacteria. The morphology of *R. mooseri* cell walls prepared by this procedure is not appreciably different from bacterial structures obtained in a similar manner. In addition to the shadowed specimens shown in this paper, Ito and Vinson (18) and Anderson et al. (4) have shown with ultrathin sections that *R. quintana, R. prowazekii, R. rickettsi, R. sennetsu*, and *R. tsutsugamushi* possess a three-layered outer limiting membrane similar to that found in bacteria (16).

The chemical composition of *R. mooseri* cell walls resembles that of the gram-negative bacteria, *E. coli* (45), and *Brucella abortus* (24, 30). The cell walls of psittacosis group agents are chemically similar (though lacking DAP) to those of rickettsiae and of gram-negative bacteria, but differ in their shape and ease of preparation from intact organisms (20). The detection of DAP in typhus rickettsiae and the localization of this compound to the cell wall, in addition to the presence of muramic acid (1) already reported, suggest the existence of mucopeptide structures similar to those found in bacterial walls.

Limitations in the amount of material available for chemical analyses precluded the study of lipid material in this organism, although the presence of a considerable amount has been reported by others (9). This lack of information limits comparison of this organism to gram-positive or gram-negative bacteria. Ancillary evidence, however, has led us to the conclusion that typhus rickettsiae bear strong similarities to gram-negative bacteria: (i) the gram-negative reaction of these organisms in the traditional Gram stain (17); (ii) the presence of more than four or five amino acids in the cell wall; (iii) the demonstration by Olitski et al. (27) and Wisseman et al. (*in preparation*) that typhus rickettsiae, as well as their cell walls, induce physiological responses in animals indistinguishable from those produced by the endotoxin of *E. coli*; and (iv) the absence of teichoic acid-like compounds, eliminating a number of the gram-positive bacteria from consideration.

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