Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 36 (1979)
Heft: 2

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DOI: https://doi.org/10.5169/seals-312514

Acta Tropica *36*, 123–131 (1979)

Isolation of the outer envelope, chemical components, and ultrastructure of *Borrelia hermsi* grown in vitro

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In memoriam Oscar Felsenfeld

Summary

Gram amounts of *B. hermsi* cells were obtained from 3 liter stationary batch cultures using a modified Kelly's medium incubated at 35° C. The generation time was 12.3 h and cell densities reached 2.0×10^8/ml. Treatment of *B. hermsi* with 0.005% SDS for 20 min solubilized the outer envelope while causing little disruption of the protoplasmic cylinder and associated axial filaments (flagella). Upon filtration, dialysis, and centrifugation the SDS supernatant fluid yielded a pellet which contained membrane structures, but no visible wall fragments or whole cell when examined by electron microscopy. Muramic acid and ornithine were detected in whole cells and in the protoplasmic cylinders, but not in the outer envelope preparation, suggesting that ornithine is a component of the *B. hermsi* cell wall.

The dry weight yield of outer envelope preparation was 20.9% of the whole cell. Chemical analysis revealed the outer envelope preparation was composed of 23% lipid, 62.5% protein, and 4.0% carbohydrate.

Comparison of electron micrographs of *B. hermsi* and *B. hispanica* revealed a twofold difference in the number of axial filaments (flagella) between these species. The average number of axial filaments (flagella) found per cross-section of *B. hermsi* is 10.5 and the average number found per cross-section of *B. hispanica* is 20.

Key words: Borrelia hermsi; outer envelope; muramic acid; ornithine; axial filaments (flagella); *Borrelia hispanica.*

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Introduction

The major structures shared by members of the genus *Borrelia* are readily discernable with the electron microscope. The outer envelope (OE) surrounds the coiled protoplasmic cylinder (PC) with the axial filaments (flagella) between the outer envelope and the cell-wall membrane complex (WM) of the PC. Axial filaments (flagella) originate subterminally at each end of the cell and extend toward the opposite end following the inner side of the spiral turns in the PC. The nucleoplasm is located near the center of the PC where fine nuclear fibrils characteristic of the DNA region in procaryotic cells stained with osmium tetroxide can be seen. Ribosomes are found in the cytoplasm peripheral to the centrally located DNA.

The different species of *Borrelia* are difficult to characterize taxonomically. They do not show microscopically detectable modifications, either during their individual life cycles in the vector and host, or from species to species (Geigy, 1968). Species are determined mainly by the vectors that carry them (Davis, 1948).

The ultrastructure of different species as seen with the electron microscope has not revealed distinguishing characteristics. Aeschlimann et al. (1968) found no significant ultrastructural differences between *B. duttoni, B. tillae, B. crocidurea,* and *B. hispanica* whose cross-sections each had 12–25 axial filaments (flagella). Hovind-Hougen (1976) found *B. merionesi* and *B. recurrentis* cells to be morphologically identical each having 15–20 axial filaments (flagella) inserted at both ends of the cell.

The cultivation of *B. hermsi* in artificial media was achieved by Kelly (1971) thus making available a potential source of large amounts of these cells free of contaminating vector or host components.

We have grown batch cultures of *B. hermsi* using Kelly's media which yielded gram amounts of cells for analysis. The OE was solubilized using sodium dodecyl sulfate (SDS) and reaggregated by dialysis against water. Determinations were made of the percent protein, lipid, and carbohydrate present in the *B. hermsi* cells and cell fractions. Ornithine was found in the cells and the PC fraction indicating indirectly that ornithine is a structural component of the peptidoglycan found in *B. hermsi* cell walls. A comparison was made between the ultrastructure of *B. hermsi* and *B. hispanica,* revealing a twofold difference in the number of axial filaments (flagella) between the two species as seen in electromicrographs of the cell cross-sections.

Materials and methods

Growth in animals. B. hispanica obtained from Mr. A. W. Hanson, Venereal Disease Research Laboratory, The Center for Disease Control, Atlanta, Georgia, was maintained in short hair albino guinea pigs (Gopher State Supplies, Minneapolis, MN) by syringe passage. Blood drawn by cardiac puncture into a syringe containing 2.94% sodium citrate (w/v) solution from an infected animal

during the primary borrelemia was used for intraperitoneal injection of guinea pigs. Borrelemias were detected by darkfield examination of peripheral blood taken by clipping the marginal nail vein with a scissors.

Growth in artificial media. Stock cultures of *B. hermsi* (obtained from Dr. C. D. Cox, Department of Microbiology, University of Massachusetts, Amherst, Massachusetts), were maintained in 30 ml screw cap tubes containing Kelly's medium (1971) incubated at 35° C. Sub-cultures were started each week with an initial concentration of 1×10^6 cells/ml and provided an on-going supply of logarithmically growing cells.

Large volume cultures. Flasks of 3 liter capacity were filled to the neck with Kelly's medium leaving only a small surface area in contact with the air and closed with stainless steel caps. These stationary flasks of medium were inoculated with 1×10^6 *B. hermsi*/ml and incubated at 35° C.

Enumeration of cells. Borreliae were counted by darkfield microscopy. Stoenner's method (1974) was used to count suspensions having 10^3–10^5 borreliae/ml. The Petroff-Hauser cell was used to count suspensions having 10^5–10^8 borreliae/ml.

Electron microscopy. Borrelia cells and cell fractions were fixed with 1% 0_s04 in veronal-acetate buffer (Kellenberger et al., 1958), dehydrated with ethyl alcohol and embedded in Epon 812 (Luft, 1961). Sections were cut with an LKB Ultramicrotome and mounted on parlodian covered copper grids. Two percent uranyl acetate was used to stain the sections observed in a Siemens Elmiskop 1A electron microscope.

Statistical analysis. The t-test for non-paired experiments was used to demonstrate a significant difference at the 1% level between the average number of axial filaments (flagella) found in *B. hermsi* and *B. hispanica* ($t = 8.55$).

Outer envelope preparation. Mid logarithmic phase *B. hermsi* cells were harvested by centrifugation at 39,000 *g*, then washed with and resuspended in PBS at a concentration of about 10^8 cells/ml. The resuspended borreliae were treated with 0.005% SDS for 20 min and examined by darkfield microscopy at 0, 5, 10, and 20 min after addition of SDS. PC's were harvested by centrifugation at 20,000 *g* and lyophilized after sampling for electron microscopy. The supernatant fluid was filtered through a 0.22 μm millipore membrane and dialyzed against 5 liters of distilled water at 4° C for 72 h with three changes of water to remove SDS. The dialyzed fluid was centrifuged at 40,000 *g* for 4 h. The pellet thus obtained was lyophilized after sampling for electron microscopy.

Chemical analyses. Total protein determinations were done on samples of lyophilized preparations calculated to contain 30–300 μg protein, and solubilized in IN NaOH following the Lowry method (1951). Gravimetric determinations of total lipid were carried out by extracting lyophilized samples with chloroform-methanol-water according to the method of Bligh and Dyer (1959), then drying the chloroform layer in a 110° C oven for 15 min, and weighing. Amino acids were assayed using the procedure of Peterson and Bernlohr (1970) which allows for detection of muramic acid, ornithine, and diamino-pimelic acid with quantitation to 10 n moles. 2 mg amounts of lyophilized preparations sealed in vials under nitrogen were hydrolized in 4 N HCl at 110° C for 12 h. Dried hydrolizates were suspended in diluting buffer (0.2 N sodium acetate, pH 2.2) and autoanalyzed (model 120B, Beckman Instruments Inc., Fullerton, CA).

Results

Growing *B. hermsi* in artificial media provided cells for isolation of the OE chemical analysis and electron microscopy. Differences in ultrastructure and chemical composition may aid in classifying species in the genus.

Stationary cultures of *B. hermsi* in Kelly's medium had a generation time of 13.4 h and cell densities reached 5×10^7/ml. When gelatin was excluded from Kelly's medium growth also occurred, and the cells had a generation time of 12.3 h with cell densities reaching 2×10^8/ml. The cellular arrangement

Table 1. Lyophilized dry weight yields of *Borrelia hermsi* cells, protoplasmic cylinders, and outer envelope preparations

Preparation	Dry weight in g/l	% of cell
Cells	0.162	100.0%
PC	0.110	67.8%
OE	0.034	20.9%

Table 2. Chemical composition of *Borrelia hermsi* cells, protoplasmic cylinders, and outer envelope preparations

Component	% of total dry weight		
	Cells	PC	OE
Lipid	26.4	28.0	23.0
Protein	38.0	35.0	52.5
Carbohydrate	14.0	18.0	4.0

changed with the increasing density of the culture. Single cells predominated in cultures of a density up to 1×10^6/ml while cultures with greater cell density had more long chains of cells (2–10 cells in length) connected end to end. Sharp bending occurred at the points of connection with each cell oriented along its own axis.

Exposure of *B. hermsi* to SDS caused disruption of the cells which was proportional to the concentration of SDS. Darkfield examination of cells after treatment with 0.05% SDS revealed that chains of motile cells had been broken into non-motile fragments. Treatment with 0.01% and 0.025% SDS resulted in chains being broken into individual non-motile cells of reduced refractility. The lowest concentration of SDS used (0.005%) broke the motile cell chains into individual cells of slightly reduced refractility. This concentration was optimal for removing the OE with minimal disruption of the PC (Figs. 1, 2). PC's with exposed, but closely associated, axial filaments (flagella) can be seen in oblique and cross-sections.

Batch cultures (each 3 liters in volume) of *B. hermsi* in Kelly's medium were inoculated and grown out under identical conditions for use in determining dry weight yields and the chemical composition of the cells, PC's and OE. Dry weight yields are shown in Table 1 and the chemical composition in Table 2. Amino acid analysis of hydrolyzed samples was done for those amino

Fig. 1. *Borrelia hermsi* protoplasmic cylinders obtained after 20 min treatment with 0.005% SDS ($\times$200,000). F = axial filaments (flagella); PC = protoplasmic cylinder.

Fig. 2. Reaggregated outer envelope fragments after dialyzing against distilled water for 72 h ($\times$200,000).

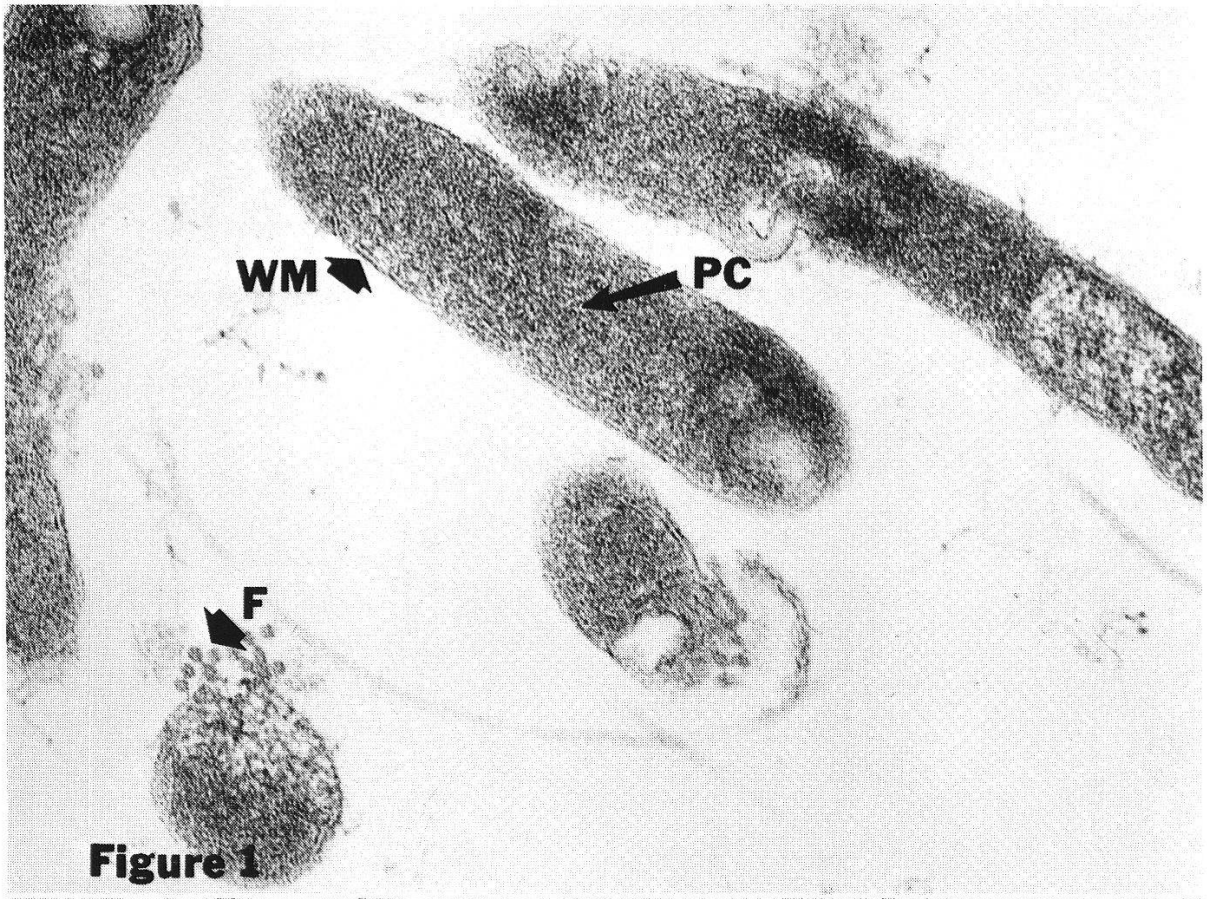

Figure 1

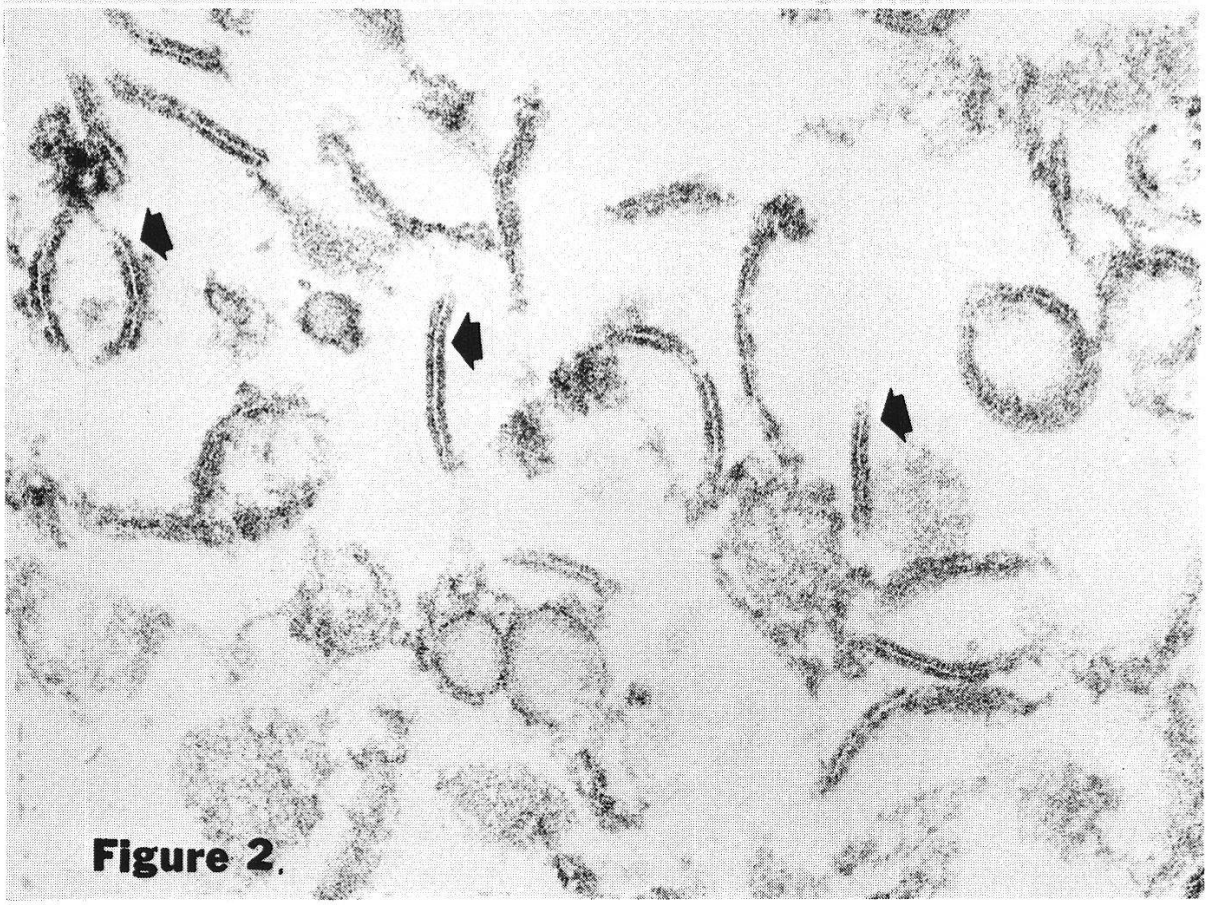

Figure 2.

Table 3. Percent total moles of amino acids in *Borrelia hermsi*

	Cells	PC	OE
Aspartic	11.74	11.21	8.51*
Threonine	5.37	5.75	5.01
Serine	6.92	7.22	5.06
Glutamic acid	11.81	10.04	11.53
Proline	5.14	4.89	10.41*
Glycine	10.92	10.70	17.54*
Alanine	12.05	11.41	10.70
Half cystine	0.55	0.81	2.61*
Valine	4.72	4.62	4.25
Methionine	N.D.	1.07	0.62
Isoleucine	3.63	3.74	1.83*
Leucine	7.77	7.86	6.44
Glucosamine	0.61	1.00	0.42
Galactosamine	0.13	N.D.	0.34
Tyrosine	2.39	2.45	2.49
Phenylalanine	3.30	3.45	4.26
Lysine	7.59	7.50	1.49*
Histidine	1.23	1.03	1.78
Arginine	2.70	2.78	3.87

* Note the difference in amount of these amino acids found in the outer envelope preparation

acids commonly found in the cell wall and other proteins. Muramic acid and ornithine were present in both the cells and PC fraction but not in the OE fraction. This indicates a good separation of the cell wall from the OE. Diaminopimelic acid (DAPA) was not detected in the cells or either cell fraction. The other amino acids detected are listed in Table 3 as the percent of the total moles of amino acids present.

Examination of electron micrographs of *B. hermsi* and *B. hispanica* revealed a difference in the number of axial filaments (flagella) between the two species. The range and average value for the number of axial filaments (flagella) found per cross-section obtained by counting them in 30 *B. hermsi* cross-sections were 4–16 and 10.5. The range and average value from 15 *B. hispanica* cross-sections were 13–26 and 20 axial filaments (flagella) (Figs. 3, 4).

Discussion

Isolation of the outer envelope from *B. hermsi* is done by solubilization with 0.0055% SDS and reaggregation by dialysis against distilled water. Mg^{++}

Fig. 3. *Borrelia hispanica.* Cross-section (×300,000). OE = outer envelope; F = axial filaments (flagella); PC = protoplasmic cylinder; WM = cell wall-membrane complex.

Fig. 4. *Borrelia hermsi.* Cross-section (×300,000). OE = outer envelope; F = axial filaments (flagella); PC = protoplasmic cylinder; WM = cell wall-membrane complex.

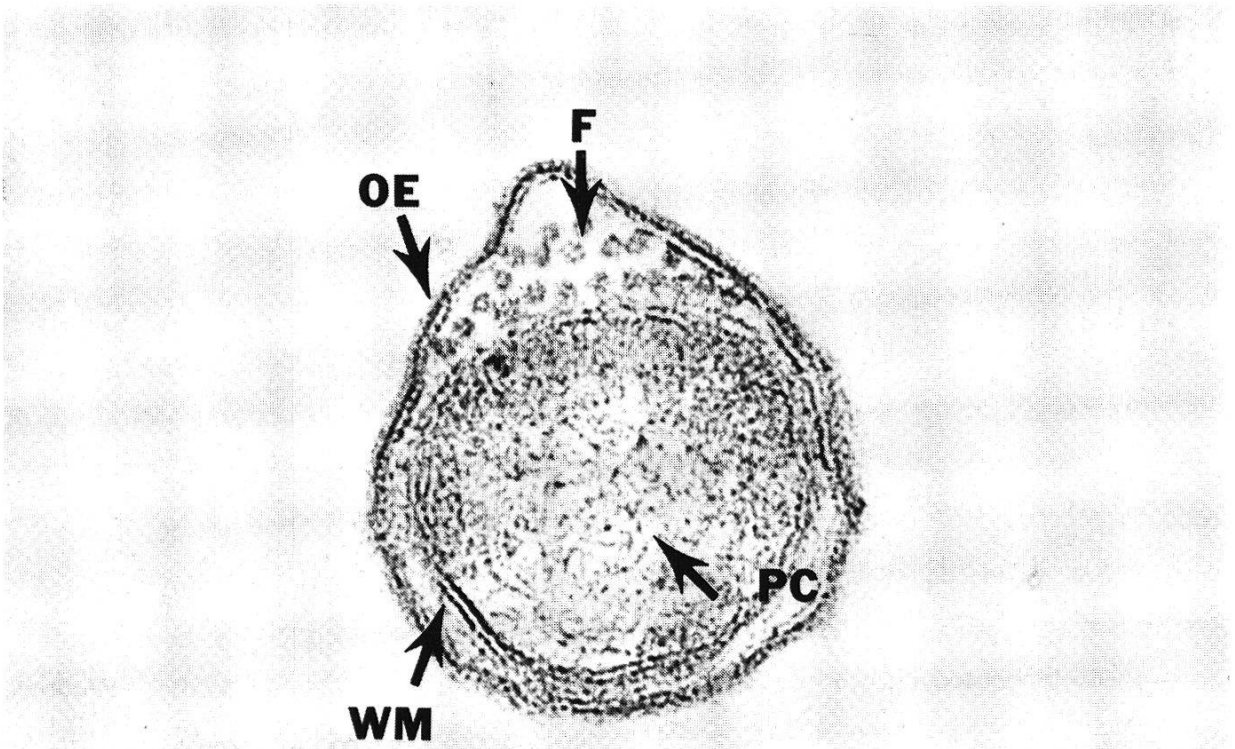

Figure 3

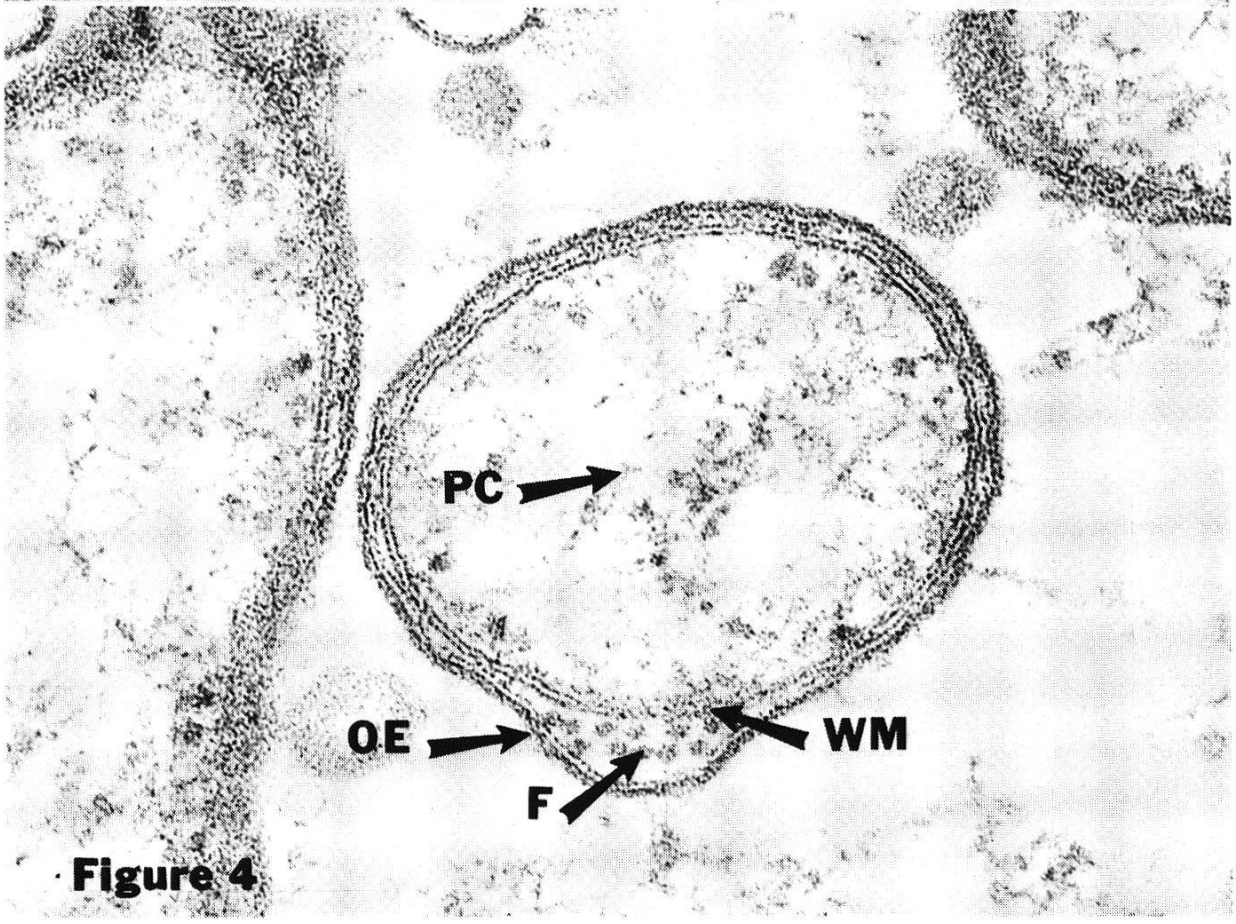

Figure 4

in other than trace amounts is not required for reaggregation. By comparison, exposure of the avirulent Kazan 5 treponeme to 1.4 mM SDS results in solubilization of its outer envelope (Johnson et al., 1973). Isolation of outer envelope from *Leptospira interrogans* serotype *canicola* Hond Utrecht IV, however, is dependent on prior separation of the outer envelope from the cell wall-membrane complex. This separation is accomplished by inducing spherical forms with IM NaCl. Subsequently, the outer envelope is solubilized with 0.02% SDS (Auran et al., 1972). The presence of Mg^{++} was not required during dialysis to reaggregate outer envelope from *Leptospira interrogans* serotype *canicola* Hond Utrecht IV (Auran et al., 1972). In contrast, Mg^{++} was required for maximal reaggregation of the outer envelope preparation from the avirulent Kazan 5 treponeme (Johnson et al., 1973). *B. hermsi* like other spirochaetes is very sensitive to the action of surface agents. Exposure to SDS concentrations higher than 0.005% results in disaggregation of both the outer envelope and the cell wall-membrane complex of the protoplasmic cylinder.

The cell wall amino acids detected in *B. hermsi* whole cells were muramic acid and ornithine. Suggesting that ornithine is present in the cell walls of the *Borrelia.* Qualitative studies on cell walls of *Treponema reiteri* have revealed ornithine to be the major diamino acid (Schleifer and Kandler, 1972). Studies on the ultrastructure and chemical composition of the cell wall of *Spirochaeta stenostrepta* have confirmed L-ornithine is a constituent of the peptidoglycan (Smibert, 1973). *Leptospira* have diaminopimelic acid rather than ornithine in their cell walls (Johnson, 1976). Amino acids other than cell wall amino acids are present covering the entire spectrum of what is generally found in bacterial cells. This method for selective removal of the OE should facilitate the chemical and immunological analysis of the borrelia.

The twofold difference in the number of axial filaments (flagella) found between *B. hermsi* and *B. hispanica* maybe a useful taxonomic tool. While no significant ultrastructural differences were noted between *Borrelia* sp. from Europe and Africa (Aeschlimann et al., 1968; Hovind-Hougen, 1976) it is possible that they differ from *Borrelia* found in North and South America. Other species of *Borrelia* found in North and South America should be compared on the basis of ultrastructure to those from Europe and Africa.

Acknowledgments. I would like to thank Ms. Donna Ritzi for her help with the electron microscopy, and Mr. David Peterson for the amino acid analyses. This investigation was supported by Public Health Service grant AI 08124 from the National Institute of Allergy and Infectious Diseases.

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