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Treponeme Outer Envelope: Chemical Analysis (39151)

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The outer envelope (OE) of *Treponema* is a membranelike structure that surrounds the flagella (axial filaments) and protoplasmic cylinder (PC). Morphological and chemical studies (1; 2; J. Pillot, Ph.D. Thesis, Univ. of Paris, 1965) have shown that the OE is a triple-layered structure that has a complex chemical structure including proteins, lipids, and carbohydrates. It is resistant to lysozyme but sensitive to a variety of lipid solvents and surface-acting agents, including sodium dodecyl sulfate (SDS).

The OE of *T. pallidum* and other parasitic spirochetes appears to play an important role in the host–parasite interaction. Infected hosts produce antibodies that immobilize or kill the infecting spirochete *in vitro* in the presence of complement, and in parasitic leptospires it has been demonstrated that the site of antibody–complement action is the OE (3). The OE of parasitic leptospires is an easily extractable immunogen (4). Animals vaccinated with purified leptospiral OE are immune to challenge (4, 5) and possess antibodies that are leptospiricidal and protective.

It is not yet known if the OE of *T. pallidum* plays a similar protective role in syphilitic infections, but if so, then the potential exists for the development of an OE vaccine for syphilis. Cultural techniques are not presently available for the *in vitro* propagation of *T. pallidum*, but it is possible to study the nonvirulent cultivatable treponemes in order to learn more about the physical, chemical, and antigenic nature of treponemal OE. A method for the solubilization and reaggregation of the OE of *T. phagedenis* biovar Kazan 5 has been described previously (6). In the present study, OE was obtained by this method from *T. phagedenis* biovar Kazan 5 and the chemical composition of the reaggregated OE was studied.

Materials and methods. Preparation of OE. Cultures of *T. phagedenis* biovar Kazan 5 were incubated for 36–48 hr at 35° in an anaerobic prereduced medium (6). The medium was modified by omitting $NaHCO_3$ and substituting the following for rabbit serum: 1% bovine serum albumin (Pentex Inc., Kankakee, Ill.) extracted three times with chloroform–methanol (2:1, v/v) for removal of lipids; 2×10^{-4} *M* palmitic acid; 2×10^{-4} *M* oleic acid; 0.25 g/liter of $CaCl_2$; 0.25 g/liter of $MgCl_2$; and 5 ml/liter of a vitamin stock solution containing (mg/100 ml) *p*-aminobenzoic acid, 100; biotin, 0.1; choline chloride, 500; folic acid, 1.0; inositol, 500; nicotinic acid, 10; pyridoxal hydrochloride, 50; pyridoxine hydrochloride, 10; riboflavin, 10; thiamine hydrochloride, 10; calcium pantothenate, 10; and vitamin B_{12}, 0.1.

Cells were harvested by centrifugation and washed once in distilled water. An aliquot of washed cells was lyophilized to determine the whole cell dry weight per liter. The remaining cells were suspended in a small volume of distilled water to which was added 250–300 ml of 0.7 m*M* sodium dodecyl sulfate (SDS) per cell mass obtained from 1 liter culture. This concentration of SDS (0.7 m*M*) effectively solubilizes the treponemal OE as well as the 1.4 m*M* SDS originally used (6), yet leaves the protoplasmic cylinder (PC) intact as shown by electronmicroscopy (6). The OE-containing supernatant was separated from the PC by centrifugation for 90 min at 16,000*g* and then filtered through a 0.45-μm membrane filter (Millipore Corp.). Reaggregation of the OE was accomplished by sequential dialysis against 4 liter volumes of distilled water (36–48 hr), 20 m*M* $MgCl_2$ (18–24 hr), and distilled water (24–48 hr) with two to three changes of each. The resulting OE aggregate was removed by centrifugation,

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washed once in distilled water, and lyophilized.

Chemical assays. Magnesium content was measured with a Perkin–Elmer Model 305-A atomic absorption spectrophotometer equipped with an HGA-70 graphite furnace. Protein content was determined by the Lowry method (7). Samples were run in duplicate on at least two occasions. Crystalline bovine albumin (Pentex Inc., Kankakee, Ill.) served as the standard and readings were made on a Beckman DU spectrophotometer at 500 nm. Determination of carbohydrate content was made according to the anthrone reagent method described by Herbert *et al.* (8), using glucose as a standard. Readings were made on a Klett–Summerson colorimeter with a 640- to 700-nm filter. Methods for the gravimetric determination of total lipid as well as for the identification of glycolipid and phospholipid components are described by Livermore and Johnson (9). Acid hydrolyses of the OE and whole cell (WC) were carried out under vacuum after flushing with nitrogen. Sealed samples were heated at 110° in either 4 *N* HCl for 4 hr or 8 *N* HCl for 18 hr. The acid was driven off with a rotary evaporator, and the samples were analyzed on a Beckman amino acid analyzer Model 120-B. A single-column methodology was employed using DC-1A resin and Pico-Buffer System II (10). Known standards were analyzed under the same condition, and recovery ranged from 75–100%, including very labile compounds such as muramic acid.

Results and discussion. An average cell yield of 600 mg (dry weight) of treponemes was obtained per liter of culture. After SDS treatment and dialysis against 20 m*M* $MgCl_2$, the OE aggregate recovered averaged 14.6% of the whole cell dry weight, or nearly 90 mg/liter of cells.

Since the reaggregation of the OE occurs in the presence of a relatively high concentration of magnesium, there was some question as to how much of the OE yield could be attributed to magnesium content. The magnesium content of the OE was found to be 0.683 μg/mg OE (Table I), or less than 0.1% of the total OE weight. As seen in Table I, the magnesium content of the reaggregated OE is very similar to the value determined for the reaggregated cell membrane of *Mycoplasma laidlawii* strain B. Both the treponeme and mycoplasma structures require the presence of magnesium ion for reaggregation (6, 11), which suggests that magnesium may be integrally bound in the membrane structure. It is of interest to note that the magnesium content of *Leptospira* outer envelope, which does not require the presence of magnesium for reaggregation (4), is much lower than that of the magnesium-dependent *Treponema* and *Mycoplasma* structures.

Like most other bacteria, treponemes contain a peptidoglycan component which helps maintain the cell shape. It has been shown that peptidoglycan extracted from the Reiter treponeme contains muramic acid, glucosamine, and other components commonly found in bacterial peptidoglycan (12, 13). More unusual, however, was the finding that the diamino acid constituent of the peptidoglycan is ornithine rather than the more commonly found diaminopimelic acid (DAPA). These findings are supported by the results of amino acid analysis of hydrolized OE shown in Table II. The presence of ornithine in treponemal peptidoglycan may be useful taxonomically since it is thought that the distribution of diamino acids in bacterial peptidoglycan may have taxonomic significance (14, 15). Of the other genera of the order *Spirochaetales*, *Spirochaeta* also has ornithine in place of DAPA (16) while *Leptospira* contains DAPA and no ornithine (12; N.E. Auran, unpublished data).

Treponemal peptidoglycan is separate from the OE and is located beneath the OE in the cell wall–cytoplasmic membrane complex of the PC (J. Pillot, Ph.D. Thesis,

TABLE I. MAGNESIUM CONTENT OF REAGGREGATED SPIROCHETAL OE AND MYCOPLASMA MEMBRANE.

	Mg^{2+} (μg/mg)
Kazan 5 (OE)	0.683
Mycoplasma laidlawii[a] strain B (membrane)	0.742
Leptospira (OE)[b]	0.197

[a] Derived from data in Ref. 18 and 19.
[b] Unpublished data.

Univ. of Paris, 1965), which is more resistant than the OE to low concentrations of SDS. The PC maintains its spiral shape and appears intact electronmicroscopically (6) after 1.4 m*M* SDS treatment for removal of OE. In the present study, analyses of acid hydrolysates of the whole cell (WC) and the OE provide further evidence of the "selective" solubilization of the OE by treatment with a low concentration (0.7 m*M*) SDS. As seen in Table II the OE is essentially free of such peptidoglycan components as muramic acid and ornithine, which are present in the WC. Amino acid profiles for both OE and WC are otherwise very similar, containing a full range of common amino acids. The OE as well as the WC contain glucosamine and galactosamine since these hexosamines are present in but not limited to the peptidoglycan structure.

Results of protein, carbohydrate, and lipid analyses of OE and WC are compared in Table III. The protein content of the OE and WC is similar, but more carbohydrate and lipid are found in the WC than in the OE.

The lipid composition of the OE is very much the same as that previously reported for the Kazan 5 treponeme whole cells (9, 17). The lipid consists of 90–95% polar lipid and 5–10% free fatty acid. The polar lipid contains 43% monogalactosyldiglyceride and 57% phospholipid, predominantly phosphatidylcholine with a small amount of phosphatidylethanolamine (17). The fatty acids present are oleic acid (30–40%) and palmitic acid (60–70%). Since the Kazan 5 treponeme cannot synthesize or degrade fatty acids (17), the lipids of both OE and WC contain only those fatty acids which are added to the culture medium.

The carbohydrate content of the OE is predominantly galactose. The galactose found in the glycolipid accounts for essentially all of the carbohydrate detected by the anthrone carbohydrate assay. Glucose

TABLE III. CHEMICAL COMPOSITION OF OE COMPARED TO WC.

	OE (%)	WC (%)
Protein	60–73	61–68
Carbohydrate	1–2	7–9
Lipid	4–5	18–20

TABLE II. ANALYSIS OF OE AND WC SAMPLES AFTER ACID HYDROLYSIS.[a, b]

	Nanomoles per milligram		Percentage of total moles	
	OE	WC	OE	WC
Aspartic acid	560	424	10.4	10.1
Threonine	297	234	5.5	5.8
Serine	231	178	4.3	4.2
Muramic acid[c]	N.D.[d]	13	N.D.	0.4
Glutamic acid	599	450	11.1	10.7
Proline	226	163	4.2	3.9
Glycine	463	402	8.6	9.6
Alanine	474	395	8.8	8.4
Half cystine	39	29	0.7	0.7
Valine	367	290	6.8	6.9
Diaminopimelic acid	N.D.	N.D.	N.D.	N.D.
Methionine	134	98	2.5	2.4
Isoleucine	372	296	6.9	7.0
Glucosamine[c]	12	25	0.3	0.7
Galactosamine[c]	25	52	0.6	1.5
Tyrosine	140	102	2.6	2.4
Phenylalanine	233	186	4.3	4.4
Ornithine	Trace	23	<0.1	0.5
Lysine	446	334	8.3	8.0
Histidine	118	77	2.2	1.8
Arginine	247	160	4.7	3.8

[a] Data represent the average of two separate analyses of each sample.
[b] Hydrolyzed in 8 *N* HCl for 18 hr unless otherwise noted.
[c] Hydrolyzed in 4 *N* HCl for 4 hr.
[d] N.D. = not detected.

is also present in very small amounts as glucosamine, which would not be detected by the anthrone reaction, and it is the only other sugar that was present in OE.

Summary. The chemical composition of the outer envelope (OE) of *Treponema phagedenis* biovar Kazan 5 was investigated. After cultivation in a lipid-defined medium, the OE was removed from the cells with 0.7 m*M* sodium dodecyl sulfate. The solubilized OE was reaggregated by dialysis against 20 m*M* $MgCl_2$, washed, lyophilized, and subjected to chemical analysis. The average yield of OE was 14.6% of the whole cell (WC) dry weight. The magnesium content was 0.683 μg/mg OE. Peptidoglycan components such as muramic acid and ornithine were detected in the WC but not in the OE, and diaminopimelic acid was absent in both WC and OE. The OE contained protein (60–73%), carbohydrate (1–2%), and lipid (4–5%), primarily polar lipid. The major polar lipids were monogalactosyldiglyceride (43%) and phospholipid (57%), of which phosphatidylcholine was the main phospholipid component, with phosphatidylethanolamine present in lesser amounts.

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