# FATE OF THE CELL WALL OF SALMONELLA TYPHIMURIUM UPON INGESTION BY THE CELLULAR SLIME MOLD: POLYSPHONDYLIUM PALLIDUM*

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The cellular slime molds have been the subject of increasing interest in the study of growth and differentiation. The vegetative form is a phagotrophic myxamoebae which normally grows on bacteria, although one species, Polysphondylium pallidum, can grow on a defined medium (Sussman, 1963). A wide variety of organisms can be utilized by the myxamoebae for growth (Raper, 1937) and growth can also be maintained on killed bacteria. The whole bacterium is engulfed and digested within cytoplasmic vacuoles (Raper, 1937; Mercer and Shaffer, 1960; Gezelius, 1961). When the bacterial supply is exhausted, the myxamoebae stop growing logarithmically and, on a solid substrate, go through the morphogenetic sequence of aggregation, pseudoplasmodium formation, and fructification.

The present investigation was undertaken to determine the fate of the cell wall of gram-negative bacteria upon ingestion by the myxamoebae. It seemed likely that they would possess enzymes capable of degrading

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the bacterial cell wall macromolecules to components which could be utilized in the synthetic processes of the dividing myxamoebae.

Myxamoebae of Polysphondylium pallidum[1/] were maintained axenically in Sussman's Defined Medium (Sussman, 1963). For the experiments Salmonella typhimurium was chosen as the bacterial associate because of the extensive chemical characterization of its cell wall lipopolysaccharide (Osborn et al., 1964). The availability of mutant organisms permitted the specific labeling of some of the monosaccharide components of the cell wall heteropolymer. In this way, degradation and reutilization of the cell wall could be detected by following the incorporation of the ingested radioactivity into various cellular constituents. If the polysaccharide moiety of the cell wall lipopolysaccharide remained unaltered it should be possible to recover the intact polymer with the same specific label. Upon acid hydrolysis, the $^{14}C$-labeled monosaccharide would be quantitatively released.

A mutant strain of S. typhimurium, which lacks the enzyme uridine diphosphogalactose 4-epimerase (Osborn et al., 1962), was grown in proteose peptone medium at 37° until just out of logarithmic growth phase. Galactose-1-$^{14}C$ was then added with an equal volume of fresh media and the culture incubated for an additional 90 minutes. Under these conditions, more than 80% of the $^{14}C$-galactose which was taken up by the cells was incorporated as galactose into the cell wall lipopolysaccharide. The washed, autoclaved bacterial cells from one liter of culture were suspended in 100 ml of Bonner's Medium (Bonner, 1947), autoclaved again and inoculated with $10^6$-$10^7$ myxamoebae. When growth of the myxamoebae was completed

1/ Cultures generously supplied by Dr. M. Sussman, Brandeis University, Waltham, Massachusetts.

(5-7 days), more than 90% of the bacteria (as determined by microscopic examination) had been ingested. The total radioactivity of the culture was essentially unchanged. The myxamoebae (containing 10-50% of the initial radioactivity of the culture) were harvested, sonicated and the cell-free extract hydrolyzed in 1.0 N HCl for 90 minutes at 100°. Paper chromatograms of the hydrolysate were developed in butanol/pyridine/water (6:4:3) and scanned. With the unhydrolyzed extract all of the radioactivity remained at the origin, while in the hydrolyzed sample 90-100% of the radioactivity migrated as galactose. Similar treatment of the $^{14}$C-cell wall-labeled bacteria (not exposed to the myxamoebae) yielded an identical pattern. Extracts of myxamoebae grown on concentrated living or autoclaved $^{14}$C-galactose-labeled mutant bacteria in buffer (without exogenous protein, carbohydrate or co-factors) gave similar results. This eliminated the possibility that enzymes for bacterial cell wall degradation might be repressed if the myxamoebae were grown in a carbohydrate- and protein- rich medium. In addition, when P. pallidum was grown on bacteria labeled with $^{14}$C-galactose on agar plates and permitted to go through the complete morphogenetic sequence, all the retained radioactivity was present as galactose after hydrolysis. An attempt to reisolate the bacterial cell wall lipopolysaccharide from the myxamoebae (Osborn et al., 1962) is outlined in Table I. Hydrolysis and chromatography of the final aqueous phase as well as the 30,000 x g supernatant fluid yielded galactose as the sole labeled product. Again prior to hydrolysis, all the radioactivity remained at the origin; there was no evidence for degradation of the lipopolysaccharide to small oligosaccharides. About 15% of the radioactivity in the myxamoebae extracts was soluble in cold trichloroacetic acid, compared to less than 1% of the

TABLE I

Isolation of Bacterial Lipopolysaccharide from Slime Mold Myxamoebae

| | | Bacterial control | | Myxamoebae | |
|---|---|---|---|---|---|
| | | cpm | % of cell-free extract | cpm | % of cell-free extract |
| 1. | Cell-free extract | 52,000 | | 37,120 | |
| 2. | Washed 30,000 x g pellet* | 42,430 | 82 | 16,960 | 45 |
| 3. | 45% phenol extraction | | | | |
| | a) aqueous phase** | 38,000 | 73 | 13,520 | 36 |
| | b) phenol phase | 1,300 | | 700 | |

* cell wall fraction
** lipopolysaccharide fraction

A culture of myxamoebae grown on living S. typhimurium with $^{14}$C-galactose-labeled cell walls and a similarly labeled bacterial culture (bacterial control) were harvested, sonicated in Tris-KCl buffer, pH 7.5 for 30 seconds, and centrifuged at 1000 x g for 30 minutes to remove unbroken cells. The supernatant fluid was centrifuged at 30,000 x g for 30 minutes, the pellet stirred for 30 minutes at room temperature in Tris-KCl buffer and then centrifuged at 30,000 x g for 30 minutes. The final pellet was extracted twice with 45% phenol for 10 minutes at 68°. The extracts were cooled in ice, centrifuged, and separated into aqueous and phenol phases. The aqueous layer was extracted with ether to remove residual phenol. Suitable aliquots were plated on planchets, dried and counted in a windowless gas flow counter.

control bacteria[2/]. This fraction remained in solution after centrifugation at 100,000 x g for 2 hours. In high voltage electrophoresis at pH 3.4 (35 volts/cm for 1 hour) this component migrated 2-3 cm toward the anode as a single, broad peak. Acid hydrolysis followed by paper chromatography revealed only radioactive galactose.

2/ Radioactivity was measured in a windowless gas flow counter.

These results suggested that the lipopolysaccharide had not been degraded by the myxamoebae. However, since polymers containing galactose have been identified in differentiating slime mold (Sussman and Osborn, 1964), it was conceivable that galactose of the bacterial cell wall was incorporated directly into these polymers instead of undergoing further metabolism. For this reason, a phosphomannose isomeraseless mutant (Rosen et al., 1964) of S. typhimurium was utilized; this organism can not metabolize mannose, but will incorporate exogenous mannose into mannose-containing polymers. A cell-free extract of myxamoebae grown on the $^{14}C$-mannose-labelled mutant was hydrolyzed, and again paper chromatographic results indicated that all the radioactivity migrated as mannose.

In the case of cells grown on either bacterial mutant, less than half of the total radioactivity of the culture was retained by the myxamoebae after complete ingestion of the cell wall labeled bacteria. This fraction was also lost to the medium during further growth. About 70% of the radioactivity released into the medium could be sedimented at 100,000 x g for 3 hours. This material, before and after acid hydrolysis, behaved in identical fashion in paper chromatography to the fraction isolated from extracts of the myxamoebae.

Further characterization of the radioactive material liberated by the slime mold during growth on cell wall-labeled bacteria was accomplished by the use of specific antibodies to the cell wall lipopolysaccharide. The radioactive substance in the cell-free culture fluid after growth of the slime mold (Table II) reacted specifically with immune antiserum and the resultant antigen-antibody complex was quantitatively precipitated

by sheep anti-rabbit γ-globulin[3/] (Scharff et al., 1963).

Thus, chemical and immunologic studies indicate that the poly-

TABLE II

Reactivity of the Material Excreted by the Slime Mold with Antiserum to the O-Antigen of S. typhimurium

| | Antigen | Immune serum | Normal serum | Radioactivity precipitated |
|---|---|---|---|---|
| | cpm | | | % |
| "Slime-Mold" Antigen | | | | |
| 1 | 689 | + | - | 58 |
| 2 | 689 | - | + | 0 |
| 3 | 332 | + | - | 90 |
| 4 | 332 | - | + | 0 |
| 5 | 94 | + | - | 100 |
| "Salmonella" Antigen | | | | |
| 1 | 987 | + | - | 41 |
| 2 | 987 | - | + | 0 |
| 3 | 225 | + | - | 79 |
| 4 | 225 | - | + | 0 |
| 5 | 76 | + | - | 90 |

A culture of myxamoebae which had grown on heat-killed $^{14}$C-galactose-labeled bacteria, in buffer, was centrifuged at 500 x g to remove the myxamoebae. The supernatant fluid, which contained over 95% of the radioactivity of the culture, was hydrolyzed in acetic acid (pH 3.5) for 1 hour at 100°, centrifuged to remove cellular debris, evaporated to dryness in vacuo and redissolved in water ("Slime-Mold" Antigen). A control suspension of heat-killed $^{14}$C-galactose-labeled bacteria was hydrolyzed and concentrated in the same manner ("Salmonella" Antigen)[4/]. Aliquots of antigen (1-10 µl) were added to immune (Difco) or normal rabbit serum (10 µl) and incubated in 0.5 ml saline for 1 hour at 37°. Sheep antiserum to rabbit gamma globulin (0.1 ml) was then added and the reaction mixtures incubated at 37° for an additional 3 hours. The tubes were then centrifuged at 2000 rpm for 10 minutes and the precipitates washed 3 times with phosphate buffered saline. The final pellet was suspended in ethanol-ammonia, dried on a planchet and counted in a gas flow counter.

3/ Sheep anti-rabbit γ-globulin was kindly supplied by Dr. M. D. Scharff, Albert Einstein College of Medicine, New York, N. Y.

4/ The mild acid hydrolysis at pH 3.5 releases the lipid from the cell wall lipopolysaccharide leaving the polysaccharide hapten solubilized and intact.

saccharide component of the cell-wall lipopolysaccharide is not significantly altered by Polysphondylium pallidum and is excreted quantitatively into the culture medium. This is true when the myxamoebae are grown in buffer on bacteria as the sole source of nutrients, or grown in Bonner's Medium which provides additional nutrients such as peptone and glucose.

Since the complete physical characterization of these S. typhimurium lipopolysaccharides is not available, it can not be stated that they are entirely unaffected by passage through the slime mold myxamoebae. The increased percentage of soluble radioactivity not precipitated by trichloroacetic acid or sedimented by centrifugation at 30,000 x g (see Table I) in the cell-free extract of the myxamoebae may reflect some degradation of the lipid moiety of the polymer or of associated protein (s). Similarly, degradation of other macromolecular components of the cell envelope (membrane, mucopeptide) is not precluded.

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