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NATIVE AND REFORMED *MYCOPLASMA LAIDLAWII* MEMBRANES COMPARED BY FREEZE-ETCHING

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SUMMARY

1. The ultrastructure of native and reformed *Mycoplasma laidlawii* membranes was compared by the freeze-etching technique.

2. Reformed membranes were prepared by dialysis of *M. laidlawii* membrane material solubilized in sodium dodecyl sulfate against buffer containing 5 or 20 mM Mg^{2+}.

3. Both the convex and concave fracture faces of freeze-cleaved native *M. laidlawii* membranes showed large areas studded with numerous globular particles, approx. 75–150 Å in diameter.

4. The reformed membranes produced with 5 mM Mg^{2+} appeared mostly as long membranous sheets, while those produced with 20 mM Mg^{2+} usually had a vesicular appearance.

5. The fracture faces of the reformed membranes were relatively smooth and did not contain the globular particles seen in cleaved native membranes.

6. This finding indicates that the ultrastructural organization of the reformed membranes differs from that of native *M. laidlawii* membranes.

INTRODUCTION

The reassembly of lipid and protein components of detergent-solubilized mycoplasma membranes had been studied in detail[1–4]. *M. laidlawii* membranes solubilized in sodium dodecyl sulfate reaggregate to membrane-like structures on removal of the detergent by dialysis against a Mg^{2+}-containing buffer. The reformed membranes contained almost all the lipid and over 85 % of the protein of the native membrane when the concentration of the Mg^{2+} in the dialysis buffer was 20 mM (ref. 4). Negatively stained preparations and thin sections of the reaggregated membrane material showed it to consist of vesicles or membranous sheets having the same thickness and characteristic triple-layered structure of the native membrane[1–4]. Enzymic activities of the native membrane which resisted inactivation by the detergent used for membrane solubilization could be recovered in the reformed membranes[1,4].

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In spite of the similarities shown on comparison of the native and reformed membranes, a most basic question still remains to be answered: is the molecular organization of the protein and lipid in the reformed membranes identical with that of the native membrane? One way to answer this question could be based on the analysis of the reformed membranes for multienzyme activities such as those involved in electron transfer and active transport, assuming that their presence would indicate the identical spatial arrangement of enzymes in both the reformed and native membranes. Unfortunately, no information is available on this subject so far. A major difficulty impeding this type of study is the inactivation of some key enzymes, such as membrane ATPase, during the initial solubilization of the membrane by the detergent[1,4].

The new freeze-etching technique for preparing biological specimens for electron microscopy has revealed membrane structures not visible by the techniques of thin sectioning and negative staining, and it eliminates the artifacts produced by fixation, dehydration, and heavy metal staining of the specimens. Furthermore, freeze-etching provides a method of examining different planes of the membrane at moderately high resolution. It seemed, therefore, worthwhile to compare the ultrastructure of the native and reformed *M. laidlawii* membranes by this technique.

MATERIALS AND METHODS

M. laidlawii (oral strain) was grown statically in a modified Edward medium[5]. The organisms were harvested after 18–20 h of incubation at 37° and washed twice in the cold with 0.25 M NaCl.

Isolation of cell membranes

Cell membranes were isolated from the cytoplasmic constituents by osmotic lysis of the organisms[3]. The membranes were washed 3 times and resuspended in 0.01 M Tris–HCl buffer (pH 7.2).

Membrane solubilization and reformation

Sodium dodecyl sulfate was added to the membrane suspension (containing 5–10 mg membrane protein per ml) to a final concentration of 0.02 M. After 15 min incubation at 37°, the clear solution obtained was centrifuged at 35000 × *g* for 30 min to remove nonsoluble material, the amount of which was usually negligible. The solubilized membrane material was dialyzed against 1000 vol. of cold *β*-buffer (0.15 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, in deionized water adjusted to pH 7.4 with HCl; ref. 1), diluted 1:20 in deionized water. $MgCl_2$ was added to the dilute buffer to a final concentration of 5 or 20 mM. Dialysis was carried out at 4° without stirring for 4 days. The reaggregated material was collected by centrifuging the contents of the dialysis bags at 35000 × *g* for 90 min in the cold.

Freeze-etching and electron microscopy

M. laidlawii cells were prepared for freeze-etching by suspending the harvested non-washed organisms for 1 h in phosphate-buffered saline containing 20 % glycerol. Isolated cell membranes and reaggregated membrane material were either suspended in phosphate-buffered saline containing 20 % glycerol or in 0.01 M Tris–HCl buffer

(pH 7.2). Small droplets of these preparations were placed on 3-mm copper discs and were immediately frozen at $-150°$ in liquid Freon 22 cooled by liquid nitrogen. The specimens were freeze-cleaved, etched and shadowed with platinum–carbon in a Balzers freeze-etching apparatus (Balzers High Vacuum Corporation, Santa Anna, Calif.) according to the method of MOOR AND MÜHLETHALER[6]. Deep-etching of cleaved specimens was done at $-100°$ for 1 min. The platinum–carbon replicas were floated off on distilled water, cleaned with Clorox, picked up on electron microscopic grids, and examined in a Philips 200 electron microscope.

RESULTS

Morphology of freeze-cleaved M. laidlawii organisms

The ultrastructural appearance of a replica of the membranes of freeze-cleaved *M. laidlawii* cells suspended in 20 % glycerol is shown in Fig. 1. Both the convex and concave faces of the cleaved membranes contained globular particles, which measure approx. 75–150 Å in diameter. The convex fracture face contained more particles than the concave fracture face, and both fracture faces contained bare areas devoid of particles. It is not possible to perform deep-etching by suspending the intact organisms in 0.01 M Tris or distilled water because they would lyse.

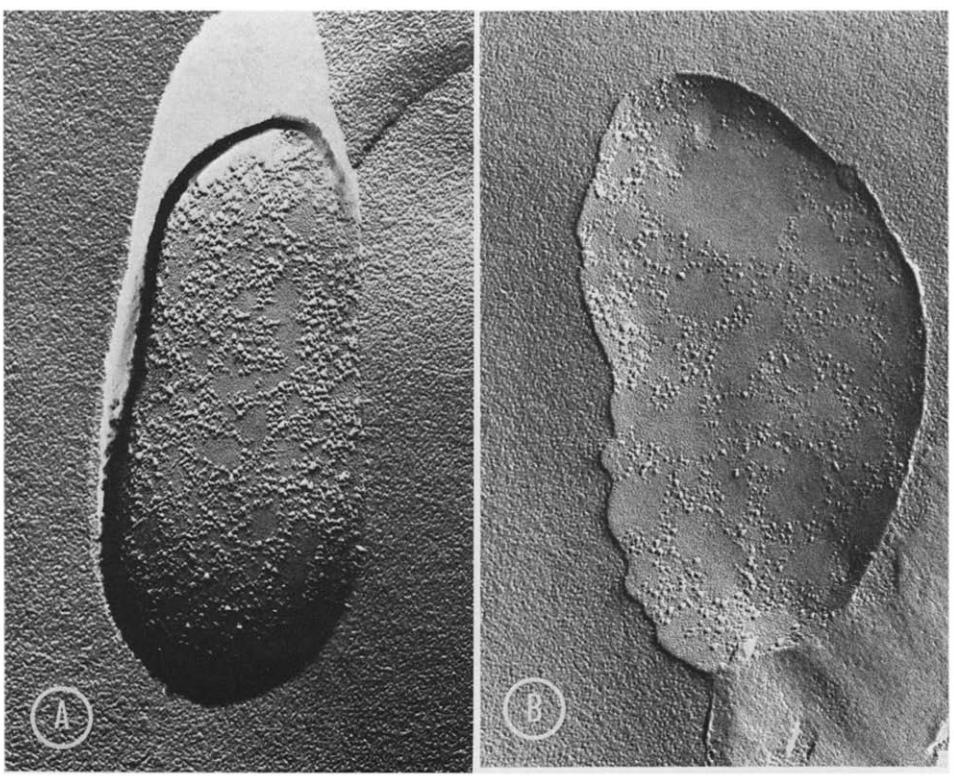

Fig. 1. Platinum–carbon replica of the convex fracture face (A) and concave fracture face (B) of the membranes of freeze-cleaved *M. laidlawii* cells suspended in 20 % glycerol. Globular particles are present on both fracture faces of the membrane. Areas free of particles can be noted. 50 000 ×.

Freeze-etched native membranes of M. laidlawii

Replicas of freeze-cleaved membranes of lysed *M. laidlawii* cells suspended in either 20 % glycerol in phosphate-buffered saline or in 0.01 M Tris are shown in Figs. 2 and 3. The number and distribution of particles on the convex and concave fracture faces of the membrane resembled those seen in the membranes of intact organisms, indicating that no obvious ultrastructural changes took place during the procedure of membrane isolation. Deep-etching of the cleaved membranes in 0.01 M Tris (Fig. 3) showed that the actual outer surface of the membrane was somewhat rough in texture, but did not contain any of the globular particles seen on the cleaved membrane faces. The inner or cytoplasmic surface of the membranes revealed by deep-etching also was free of particles but had a somewhat rough texture.

Freeze-etched reformed M. laidlawii membranes

Freeze-etch replicas of reaggregated membrane material produced by dialysis of solubilized *M. laidlawii* membranes against buffer containing 5 mM Mg^{2+} showed long membranous sheets, usually connnected to each other, forming a maze-like structure (Fig. 4). Both the convex and concave fracture faces of the reformed membranes were relatively smooth, and did not contain the globular particles seen on the cleaved faces of the native membranes. Occasional particulate structures were present on the cleaved faces of the reformed membranes, but their number and distribution were quite different than on native membranes.

The reformed membranes produced at the higher Mg^{2+} concentration (20 mM) were mostly organized as vesicles of the same size as that of the cells (Fig. 5). Some membranous sheets were also seen. Both fracture faces of the reformed membranes

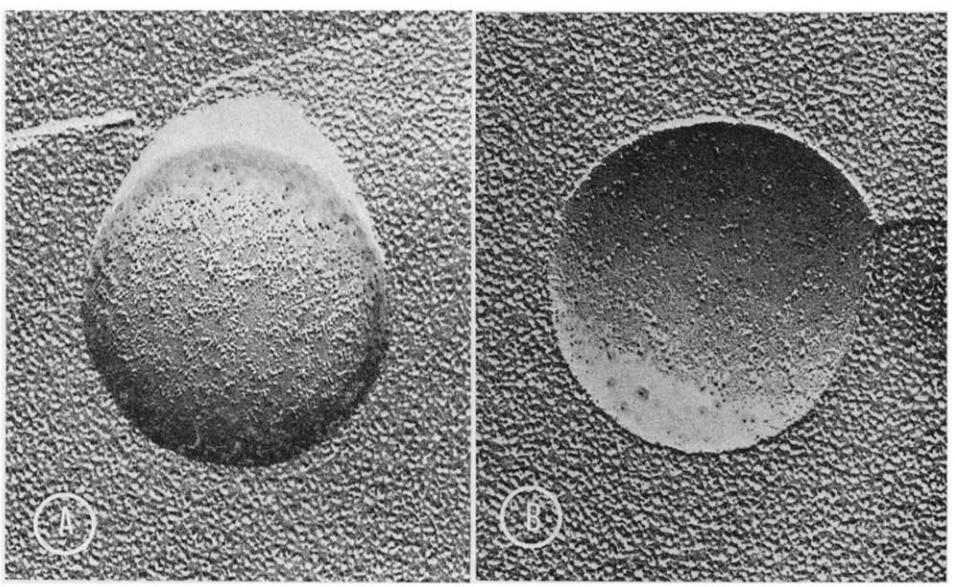

Fig. 2. Replica of freeze-cleaved native membrane vesicles of *M. laidlawii* isolated after osmotic lysis, and suspended in 20% glycerol. The convex fracture face (A) and concave fracture face (B) of the freeze-cleaved membranes show a similar number and distribution of particles as the membranes of intact organisms. 70 000 ×.

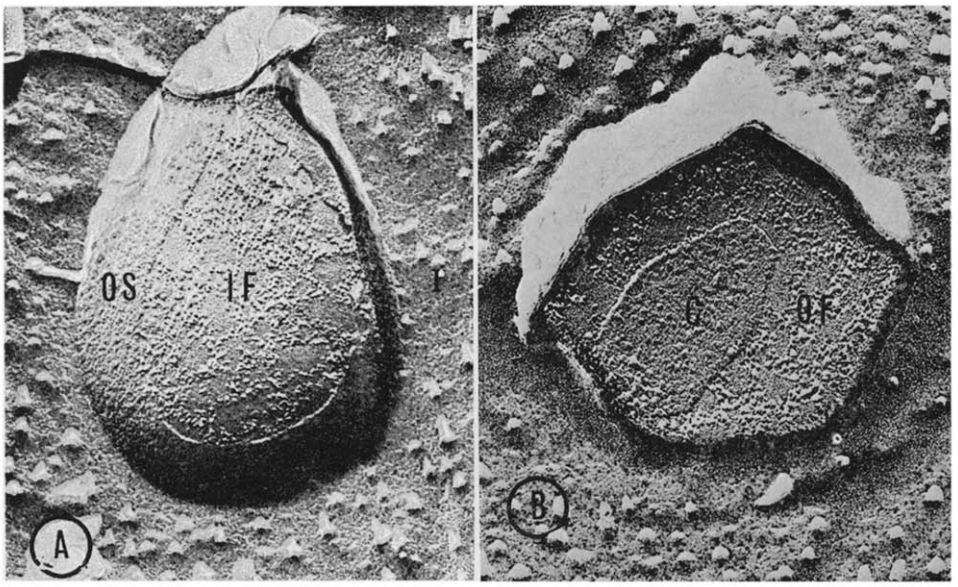

Fig. 3. Replicas of freeze-cleaved and deep-etched native membranes of *M. laidlawii* isolated after osmotic lysis and suspended in 0.01 M Tris–HCl. A. The freeze-cleaved convex (inner) fracture face (IF) contains globular particles, and sublimation of the ice (I) from the surface of the cleaved specimen has exposed the actual outer surface of the membrane (OS). 75 000 ×. B. The freeze-cleaved concave (outer) fracture face (OF) contains globular particles and the actual cytoplasmic surface of the membrane (C) is shown. 75 000 ×.

were relatively smooth and did not contain the characteristic globular particles seen on native membranes (Figs. 1–3). The actual outer and inner surfaces of the reaggregated vesicles, revealed by deep-etching, were somewhat coarse in texture, resembling the native membranes. In some preparations, a variable number of flat elevations or plaques were seen on the fracture faces of the reaggregated membranes, not unlike those occasionally seen on fracture faces of myelin, or artificial lipid bilayers[7].

DISCUSSION

The electron micrographs of the freeze-etched preparations show that the mycoplasma membrane resembles other biological membranes in containing a large number of globular particles exposed on both fracture faces of the membrane[8–10]. Recent freeze-etching studies of labeled erythrocyte membranes support the hypothesis of BRANTON[11] that the biological membrane is split by the freeze-etching process along a plane within the membrane itself, rather than along the external surface or cytoplasmic surface of the membrane[12]. The interpretation of our electron micrographs, therefore, assumes that the globular particles seen on the freeze-cleaved faces of the mycoplasma membrane are within the membrane; thus, the convex fracture face represents the inner half of the split membrane, while the concave face represents the outer half.

Particle-free areas on the fracture faces have been noticed in the *M. laidlawii*

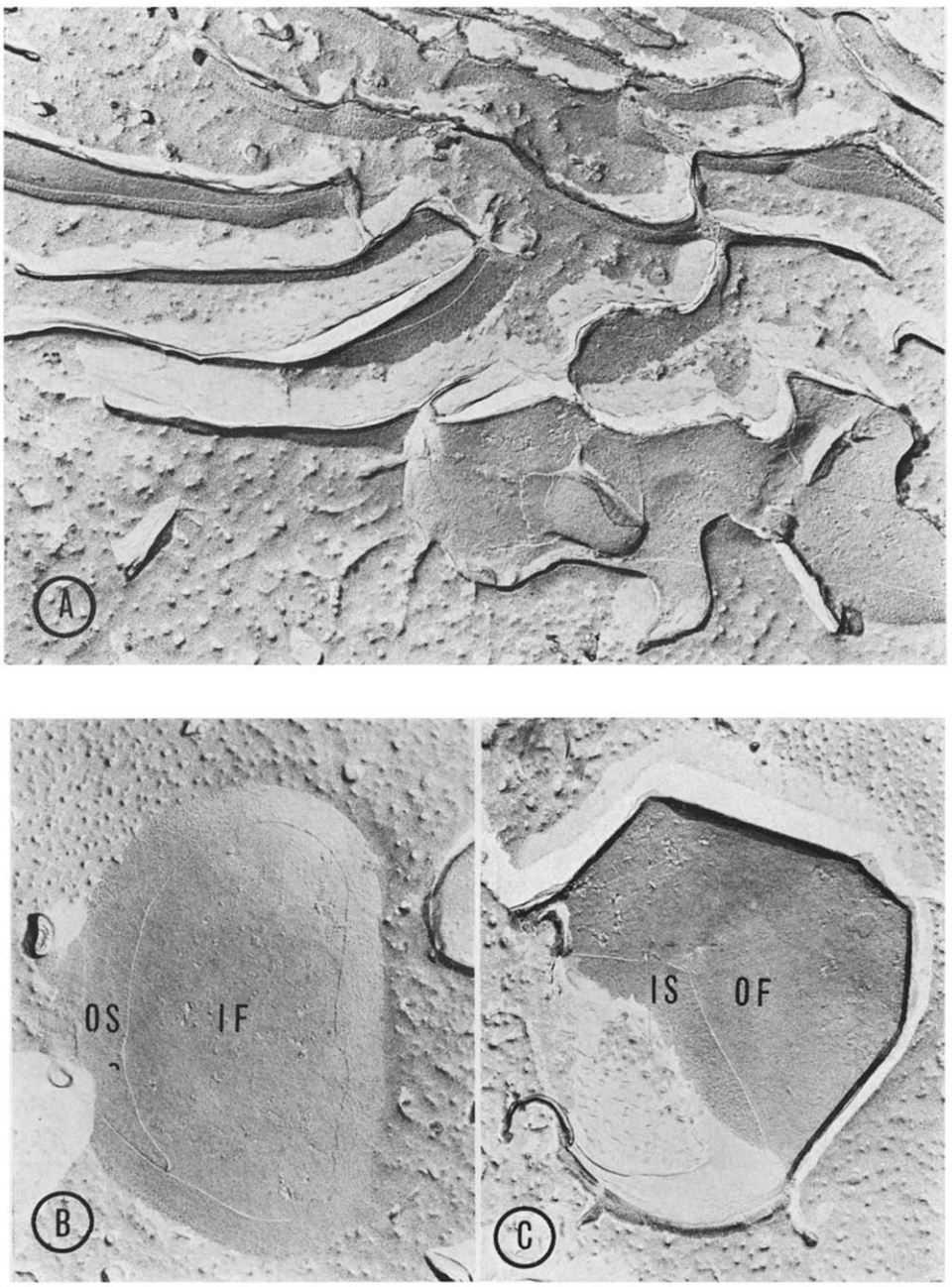

Fig. 4. Freeze-etch replicas of reformed membranes obtained by solubilization of *M. laidlawii* membranes in sodium dodecyl sulfate and dialysis against 5 mM Mg^{2+}. A. The reaggregated membrane material forms long connected sheets. 30 000 ×. B. The convex (inner) fracture face (IF) of the reformed membrane is relatively smooth, as is the actual outer surface (OS). 40 000 ×. C. The concave (outer) fracture face (OF) as well as the actual inner surface (IS) are also devoid of particles. 40 000 ×.

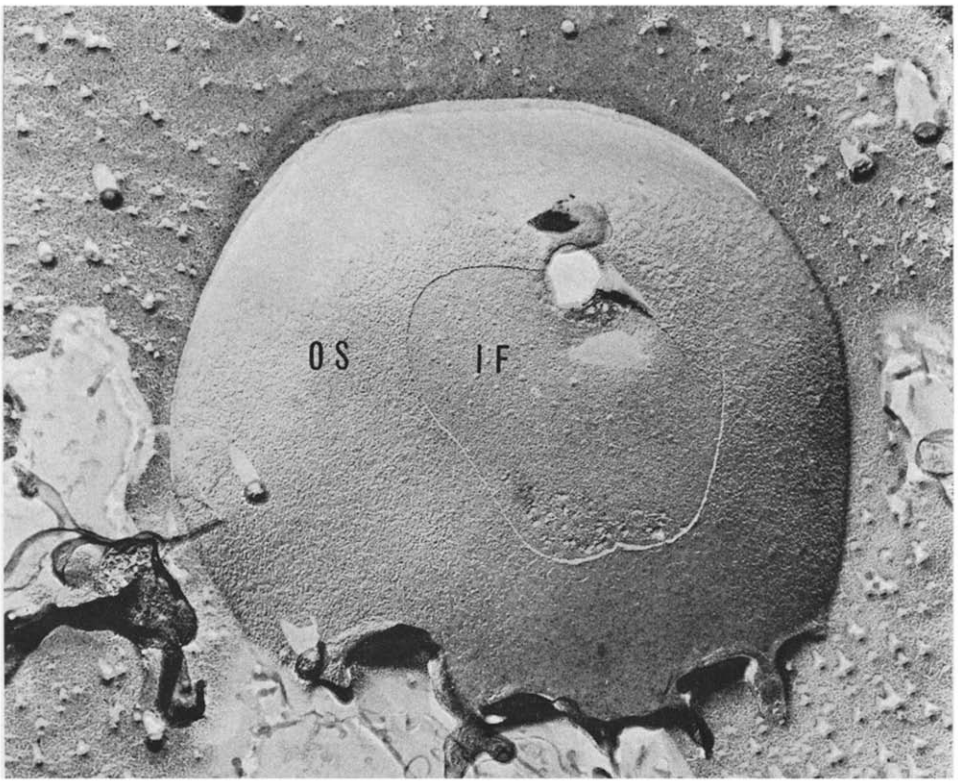

Fig. 5. Freeze-etch replica of reformed membrane obtained by solubilization of *M. laidlawii* in sodium dodecyl sulfate and dialysis against 20 mM Mg^{2+}. The reaggregated vesicular membrane has a relatively smooth convex (inner) fracture face (IF) and a smooth outer surface (OS). 45 000 ×.

membrane, resembling the particle-free areas recorded by FIIL AND BRANTON[9] and BAYER AND REMSEN[10] in *Escherichia coli* membranes. The distribution of particles on the fracture surfaces of the erythrocyte membrane is more homogeneous and does not usually show these particle-free patches[12]. The actual outer surface of mycoplasma membrane, and the inner or cytoplasmic surface of this membrane revealed by deep-etching were free of particles, but had a somewhat rough texture. This is in contrast to the outer surface of the red blood cell membrane which is smooth, and to the cytoplasmic surface of the red cell membrane, which contains rod-like structures approx. 1000 Å in length (R. E. SCOTT, T. W. TILLACK AND V. T. MARCHESI, in preparation).

The gross picture obtained by the freeze-etching technique agrees with that obtained by thin sectioning and by negative staining[3,4] in showing that the reaggregated membrane material formed at low Mg^{2+} concentration (5 mM) is organized mostly as large membranous sheets, while at higher Mg^{2+} concentration (20 mM) it is organized mostly as vesicles. The fact that the reformed membranes produced at high Mg^{2+} concentration contained almost all the protein species of the native membrane, while the reformed membranes produced at low Mg^{2+} concentration contained only a few of the membrane protein species[4], did not seem to influence the ultrastructure of these preparations as revealed by the freeze-etching technique. In

all cases the reformed membranes seemed to lack the particles characterizing the native membrane. In this respect the reformed membranes resembled the myelin membrane[13] or lipid bilayers[7]. Flat elevations or plaques could frequently be seen on the fracture surfaces of the reformed membranes, again resembling in this respect myelin and artificial phospholipid membranes[7].

Though the interpretation in biochemical terms of the ultrastructural detail revealed by freeze-etching is still uncertain, the present results seem to indicate differences in the organization of the protein and lipid between the reformed and the native *M. laidlawii* membranes.

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