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17 August 1972

## Freeze-Fractured Acholeplasma laidlawii Membranes: Nature of Particles Observed

Abstract. *Freeze-fracture of* Acholeplasma laidlawii *membranes from cells incubated in the presence of puromycin or omission of amino acids reveals a decrease in the number of particles between 50 and 100 angstroms in the hydrophobic fracture plane, which strongly suggests that these particles are protein. Additional evidence indicates that they may be involved in substrate transport.*

The freeze-fracture technique in conjunction with electron microscopy has been a unique tool for studying membrane structure. Although there has been considerable controversy (*1*) as to where the primary fracture plane in biological membranes occurs, it has been convincingly demonstrated that the major fracture plane in membranes is down the center of the hydrophobic portion of the lipid bilayer (*2*).

A most interesting structural feature revealed in these preparations is the presence of particles between 50 and 100 Å in the hydrophobic region of the fracture plane. The precise chemical nature and function of these globular intercalations, which interrupt the continuity of the membrane's lipid bilayer, has been the subject of conjecture. That these interruptions are more abundant in membranes with high metabolic activity, such as chloroplasts, bacteria, and mitochondria, and are absent in the more metabolically inert biological membranes such as myelin and in synthetic liposomes (*1*) suggests that they may play a dynamic role in membrane function.

Digestion of red blood cell membranes (*1*, *3*) by the broad spectrum proteolytic enzyme, Pronase, for 10 hours results in almost total loss of these particles in fracture faces, which suggests that they are protein. Similar digestion of *Acholeplasma laidlawii* membranes has presented inconsistent results; that is, a moderate reduction in the number of particles was observed in some experiments and little or no reduction in others. Thus, the nature of these particles in *A. laidlawii* in regard to Pronase digestion is uncertain. In addition, since Pronase has been reported to exhibit esterase activity (*4*) the possibility that particles are lipid cannot be ruled out.

In this report we present evidence, obtained by a different approach, suggesting that these particles in *A. laidlawii* are protein in nature and that at least some of them may be involved in substrate transport across the cell membrane.

*Acholeplasma laidlawii* cells were grown in lipid-poor medium supplemented with oleic acid (30 mg/liter) (*5*). At 16 hours of growth, puromycin (25 μg/ml) was added to a portion of the growing cells, and [$^{14}$C]glutamine and [$^{3}$H]palmitic acid were added to monitor protein and lipid synthesis. Controls consisted of portions of the growing cells containing radioactive glutamine and palmitic acid to which no antibiotic was added. Samples were taken at 2-hour intervals, and the membranes were prepared (*5*) and freeze-fractured in a Balzers freeze-etching device.

Isotope counting was accomplished by using a Packard Tri-Carb liquid scintillation spectrometer. Isopycnic density gradient analyses were done as described by Kahane and Razin (*6*). Incorporation of $^{14}$C-labeled thymidine, uridine, and glucosamine was monitored on separate portions of the same batch of cells used for the experiments described above. Active transport of the nonmetabolizable sugar [$^{14}$C]2-deoxyglucose was followed by methods described by Zupnik and Tourtellotte (*7*), by using cells treated as described above with the omission of radioactive glutamine and palmitic acid. The rates were determined as counts per minute per milligram of membrane lipid.

For experiments demonstrating lipid synthesis in a medium devoid of amino acids, cells were washed three times with the following buffer (buffer K): 0.1*M* tris(hydroxymethyl)aminomethane (tris), 0.2*M* KCl, 0.01*M* $MgCl_2$,

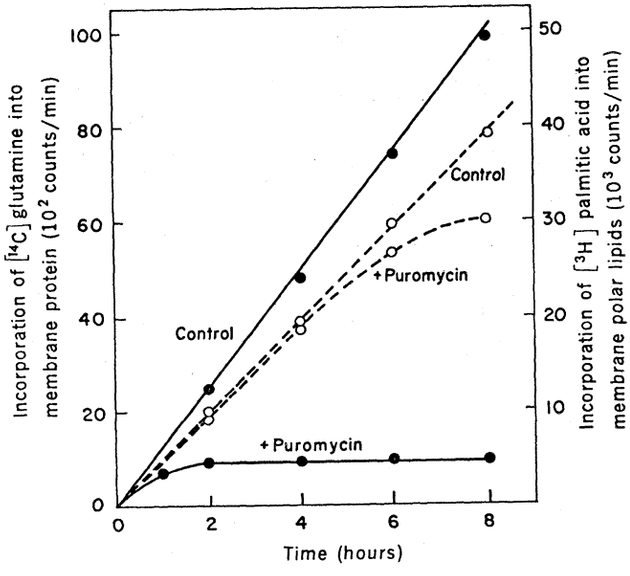


Fig. 1. Effect of puromycin on membrane lipid and protein biosynthesis in *Acholeplasma laidlawii*. (Open circles) Polar lipid synthesis; (filled circles) protein synthesis. The controls contained no puromycin.

*p*H 8.2. The incubation mixture consisted of cells; buffer K; 0.4 percent lipid-poor bovine serum albumin; 1 percent glucose; lauric, myristic, palmitic, and oleic acids (5 mg/liter); and 100 $\mu$c of [$^{14}$C]glutamine and 50 $\mu$c of [$^{3}$H]palmitic acid per liter.

As demonstrated in Fig. 1, in the presence of puromycin, polar lipid synthesis equal to that in the control containing no antibiotic continued for approximately 6 hours, dropping at 8 hours. Protein synthesis at 1 hour was essentially abolished by puromycin. As shown by Kahane and Razin (*6*), chloramphenicol inhibited total cell protein synthesis but not lipid synthesis in *A. laidlawii*, suggesting, as supported by our results with puromycin, that membrane lipid and protein synthesis are not coupled in *A. laidlawii*, but occur independently of each other. Further support for this hypothesis was obtained by the isolation of membranes from normal and puromycin-incubated cells (4 hours) and centrifugation in sucrose density gradients. Membranes from control cells had a density of 1.18 g/cm$^3$ while those from cells incubated in puromycin had a density of 1.15 g/cm$^3$; this demonstrates a higher ratio of lipid to protein in the cells inhibited by puromycin. Similar results on sucrose gradients were also observed by Kahane and Razin (*6*).

An examination of thin sections of the membranes by electron microscopy demonstrated no structural differences between 4- and 6-hour controls and puromycin-incubated cells.

However, the results of freeze-fracture, as shown in Fig. 2, demonstrated marked differences. It can be observed that the number of particles in cells incubated in the presence of puromycin are substantially decreased at 4, 6, and 8 hours under conditions where extensive lipid bilayer synthesis but no protein synthesis is occurring.

Figure 2b (cells plus puromycin, incubated for 4 hours) showed approximately a 50 percent decrease in the number of particles as compared to the 4-hour control (Fig. 2a); in Fig. 2c (6 hours) bare patches of lipid bilayer became more prominent and the number of particles was further decreased. In Fig. 2d (8 hours) extensive bare patches and aggregation of particles were observed. The expansive bare areas and aggregation of particles at 8 hours suggest that after extensive inhibition of de novo protein synthesis, protein particles within the membrane show translational freedom of movement within the lipid bilayer. The 8-hour control containing no puromycin resembled the 4-hour control.

Since other data showed that the incorporation of radioactive thymidine, uridine, palmitic acid, and glucosamine, respectively, into DNA, RNA, lipid, and polysaccharide was unaffected by puromycin, and the effect of this antibiotic on protein synthesis is well established (*8*), it is highly suggestive that the particles observed in freeze-fractured *A. laidlawii* membranes are protein.

Further evidence indicating that these particles are protein was obtained by growing cells for 16 hours in the presence of [$^{14}$C]glutamine and [$^{3}$H]-palmitic acid and incubating the cell suspensions in a medium free of amino acid and consisting of buffer K; bovine serum albumin; lauric, myristic, palmitic, and oleic acids; glucose; and [$^{3}$H]-palmitic acid and [$^{14}$C]glutamine to monitor continued lipid and protein synthesis. A doubling of lipid synthesis in 4 hours was demonstrated by the continued rapid incorporation of [$^{3}$H]-palmitic acid into polar lipids; the absence of protein synthesis was demonstrated by the lack of further incorporation of [$^{14}$C]glutamine into protein. In fact, in agreement with Kahane and Razin (*6*), a decrease in the [$^{14}$C]-glutamine and total protein in the membranes was observed, which suggests a turnover of the membrane proteins with a half-life of approximately 3 hours. Further verification that, in addition to the rapid turnover of membrane proteins, no de novo protein

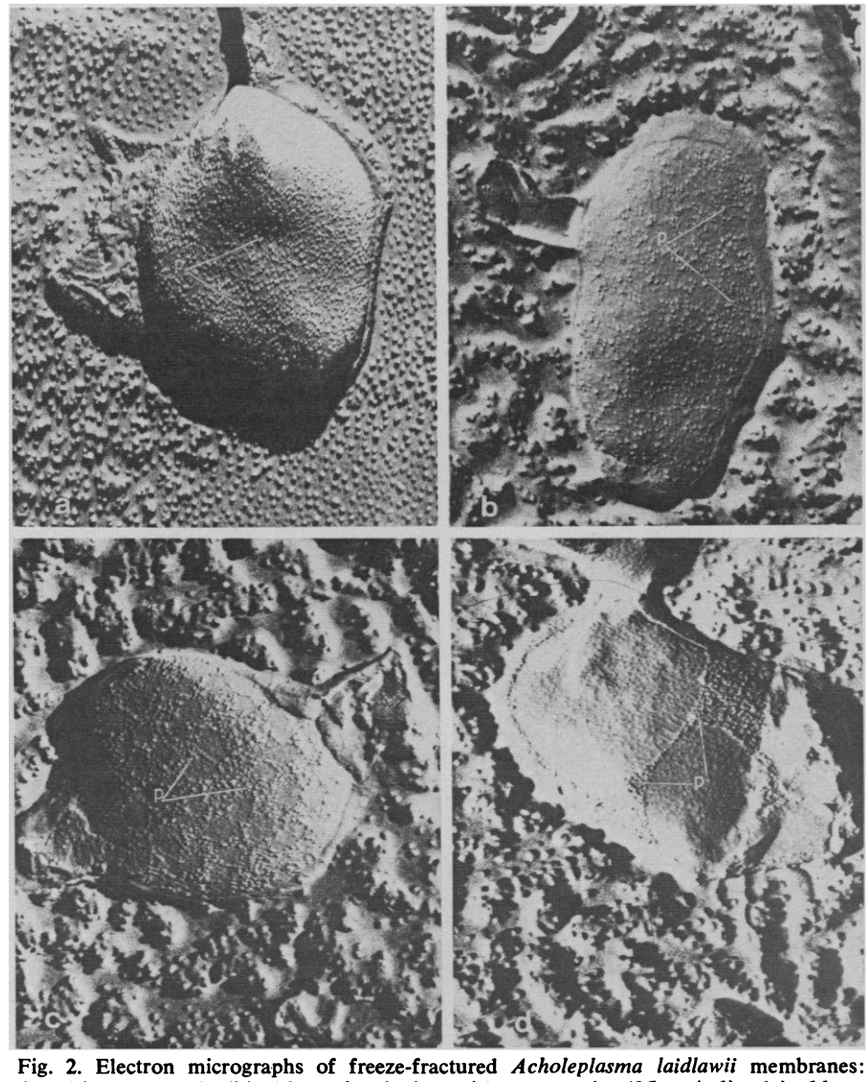


Fig. 2. Electron micrographs of freeze-fractured *Acholeplasma laidlawii* membranes: (a) 4-hour control; (b) 4-hour incubation with puromycin (25 $\mu$g/ml); (c) 6-hour incubation with puromycin; (d) 8-hour incubation with puromycin. Particles (*p*) are shown by arrows. Magnification approximately $\times$ 49,000.

synthesis occurred was obtained by using cells grown in nonradioactive media and incubating them in the media containing [$^{14}$C]glutamine and no amino acid; no significant amount of glutamine was incorporated into the cellular protein.

Freeze-fracture of cells incubated under these conditions for 2, 4, and 6 hours showed that there was a "dilution" of the number of particles in the membranes similar to that observed with puromycin. Membranes incubated under similar conditions in the presence of amino acids and tryptose resembled Fig. 2a, in which no dilution is observed. In addition, centrifugation in sucrose density gradients showed that the membranes from cells incubated in the absence of amino acids were less dense than those from cells incubated in the presence of amino acids. When cells incubated for 4 hours in a medium without amino acids, where extensive dilution of particles was observed, were transferred to a medium containing amino acids and tryptose, there was a reappearance of particles after 3 hours resembling that of Fig. 2a. These experiments support the hypothesis that these particles are protein.

The demonstration that particles observed in freeze-fractured membranes are most probably protein raises the question of the function of these protein particles. There is evidence (*7*) that deoxyglucose transport is mediated by proteins, probably consisting of subunits, which permanently traverse the membrane lipid bilayer; this suggests that at least some of the protein particles may be involved in substrate transport.

In support of this, active transport of the nonmetabolizable sugar [$^{14}$C]-deoxyglucose in cells incubated in the presence of puromycin was reduced by 50 percent at 4 hours and 85 percent at 6 hours, compared to controls. These transport results were not due to "leakiness" caused by the puromycin; there were no differences in the amounts of glycerol retention by puromycin-treated cells and untreated controls that were loaded with [$^{14}$C]glycerol, which accumulates in *A. laidlawii* by simple diffusion (*9*), and washed with cold buffer.

Since other membrane proteins, in addition to particles, are also affected under the conditions of our experiments, it cannot be stated with certainty that the particles are transport proteins; further studies are needed. However, from the evidence (*7*) and the demonstration of a reduced rate of deoxyglucose transport in puromycin-treated cells, it is tempting to suggest that at least some of these protein particles in *A. laidlawii* may be involved in substrate translocation across the cell membrane.

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## Photoreactivation of Ultraviolet-Induced Chromosomal Aberrations

Abstract. *Ultraviolet induces only chromatid-type aberrations in synchronized $G_1$ V-79 Chinese hamster and A8W243* Xenopus *tissue culture cells. Posttreatment with white light prevents expression of most potential aberrations in the A8 toad cell, which possesses a photoreactivation enzyme. We conclude that the major ultraviolet-induced DNA lesion leading to chromosomal aberrations is the pyrimidine dimer.*

Although the induction of chromosomal aberrations by ionizing radiation has been extensively investigated, there have been few studies of the induction of aberrations by ultraviolet light (UV), largely because of the difficulty of getting this radiation into the nuclei of cells of the classical plant materials (*1*). Study of aberration induction by UV is attractive because of the specific information available about the nature of UV-induced DNA lesions and their repair (*2*), and the possibility that it might provide additional information about eukaryote chromosome structure. Humphrey *et al.* and Chu (*3*) have investigated the production of chromosomal aberrations by UV in Chinese hamster tissue culture cells as a function of stage in the cell cycle. Both papers reported the production of aberrations by UV in the nucleic acid absorption range of 240 to 265 nm, and also stated that both the chromosome-type aberrations characteristically produced by ionizing radiation in cells in the pre-DNA-synthetic ($G_1$) phase and the chromatid-type aberrations produced in those in the DNA-synthetic (S) and postsynthetic ($G_2$) phases of the cell cycle could be induced by UV. Chu furthermore reported that production of UV-induced chromosomal aberrations could not be photoreactivated in the hamster cells by treatment with light in the 330 to 400 nm range.

It was subsequently discovered by Regan and Cook (*4*) that although cells from placental mammals lack any photoreactivating enzyme (PRE) activity, cells from nonmammalian vertebrates do possess demonstrable PRE activity capable of repairing UV-induced cyclobutane dimers in DNA. We were thus led to reinvestigate the question of whether eukaryote chromosomal aberration production by UV is photoreactivable in a nonmammalian vertebrate tissue culture cell line having demonstrable PRE activity. Our first experiments produced such unexpected and seemingly conclusive results that we report them here, even though our investigations are as yet incomplete.

Both a clonal derivative, A8W243, of a cell line from the amphibian *Xenopus laevis* and the V-79 Chinese hamster cell line were used for different experiments. The culture methods employed and the capacity of the A8W243 cells, but not the V-79 cells, to photoreactivate UV-induced loss of reproductive integrity have already been reported (*5*). The cell lines were synchronized by a modification of the mitotic harvest method of Terasima and Tolmach (*6*). Cell cycle analyses were done by pulse labeling with tritiated thymidine and autoradiography. Metaphases were collected with Colcemid (Ciba) and cytological preparations were made by the techniques widely employed in mammalian cytogenetics: hypotonic treat-