ULTRASTRUCTURAL ORGANIZATION OF CHLOROPLAST THYLAKOIDS OF THE GREEN ALGA *OOCYSTIS MARSSONII*

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ABSTRACT

Intact cells of *Oocystis marssonii* were thin sectioned and freeze-etched, using conventional and double-recovery techniques. Thylakoids extend the length of the single chloroplast and occur in stacks of three to five. The peripheral thylakoids in a stack often alternate between adjacent stacks. Interpretation of double-recovery results suggests that membranes in unstacked regions are asymmetrical, with one face smooth and the matching face covered with closely packed 85–90 Å diameter particles. Adjacent membranes in stacked regions evidently share 170 Å diameter particles, and either membrane in a stacked region may fracture. The two fracture planes thus made possible may expose nearly entire 170 Å particles or only the upper portion of such particles, creating in the latter case images of 125–135 Å diameter particles. Fracture planes in all cases appear to occur through the interior of the membrane, in the plane between the hydrophobic ends of the lipid bilayer proposed in numerous membrane models.

INTRODUCTION

Chloroplast membranes have proved among the most profitable subjects for freeze-etch investigations. Beginning with the work of Mühlethaler et al. (11) and others (13, 4) and continuing to the correlated physiological and ultrastructural work of Arntzen et al. (1), this membrane system has come to be one of the best characterized (see review by Kirk [7]). The aforementioned papers all deal with chloroplasts of higher plants, mostly in their isolated state.

In view of the importance of algae as experimental organisms in photophysiology, it is surprising that they have been little studied by freeze-etching (2, 5, 6, 12, 15, 17). In the only critical study of a green alga (5), Goodenough and Staehelin documented a continuum of particle sizes on each of the fracture faces of thylakoids in chloroplasts isolated from *Chlamydomonas reinhardtii*. Occurrence of the largest particles was shown to be a function of the stacking of the thylakoids.

We have looked at intact cells of the unicellular green alga *Oocystis marssonii* with conventional and double-recovery freeze-etch methods (9, 10). By using intact cells of an alga which absorbs glycerol freely we hoped to eliminate any possibility of artifacts or alterations arising during chloroplast isolation procedures. The double-recovery technique has enabled us to confirm several aspects of Goodenough and Staehelin's model and has shown the existence of a unique kind of asymmetric membrane in *Oocystis* chloroplasts. A model for the organization of the membranes is proposed to incorporate our findings and relate them to current membrane models (3, 16).

MATERIALS AND METHODS

A culture of *O. marssonii* Lemm. was obtained as no. 287 from the Culture Collection of Algae at Indiana University and grown on Bristol's medium (18) at 75°F under fluorescent illumination. Cells for ultrathin sectioning were centrifuged and fixed in 2% cacodylate-buffered glutaraldehyde at pH 7.4 at room temperature for 2 h. Postfixation was in similarly buffered 2% OsO_4 at room temperature for 1 h and embedding was in an Epon-Araldite mixture (8). Sections were poststained in uranyl acetate and lead citrate (14). Cells for freeze-etching were centrifuged and suspended for 12–18 h in 30% glycerol, frozen in Freon 22, and freeze-etched according to the methods of Moor and Mühlethaler (9), using a Balzers BA 360M machine (Balzers High Vacuum Corp., Santa Ana, Calif.). Etch times varied from 1 to 2 min.

Deposition of the standard 20 Å thick layer of platinum was checked with the Balzers quartz crystal thickness monitor, and the 40° shadow angle was kept constant. Particle sizes were measured by viewing negatives of × 118,000 original magnification at × 15 under a dissecting microscope fitted with an ocular micrometer. Measurements were made normal to the direction of shadow by measuring the platinum image at its widest point. The microscope was calibrated with a Ladd diffraction grating (Ladd Research Industries, Burlington, Vt.) at the beginning of each load of film.

Some cells were prefixed in glutaraldehyde for comparison, but images thus obtained did not differ from those of unfixed material. In addition, cells first treated with 30% glycerol were then fixed in glutaraldehyde and processed for ultrathin sectioning. No differences from cells not pretreated with glycerol were discernible. Double-recovery studies were made using the hinged device and procedures described by Mühlethaler (10). All replicas were cleaned in 50% chromic acid overnight. Sections and replicas were viewed and photographed with a Hitachi HU-11E or HU-11C electron microscope operated at 75 kV.

RESULTS

In the typical cell shown in Fig. 1, the single chloroplast fills most of the cytoplasm. Several starch grains appear in a cluster near the center of the chloroplast. The outer chloroplast membrane is relatively smooth in face view. The characteristic way in which the lamellae associate in stacks of three to five can be seen in Figs. 2 and 3. Peripheral lamellae in a stack often change association from one stack to another along the length of the chloroplast. As they do so, stretches of lamella in which there is no stacking with adjacent lamellae are created. Each lamella (Fig. 3) is a flattened sac surrounded by a tripartite unit membrane approximately 80 Å thick. In areas where two such membranes contact each other, such as between lamellae in the stack, the two membranes taken together measure about 155 Å in thickness. Such tight membrane junctions exhibit a five-layered pattern with a single thick dark center layer in the apposed regions, similar to the grana regions in higher plant chloroplasts.

When the freeze-etched lamellae are examined in detail (Figs. 4–6), four kinds of fracture surfaces appear: (1) A completely smooth surface devoid of any particulate component except for the platinum background. This surface is illustrated at high magnification in the inset of Fig. 4. (2) A surface with many pits and a few scattered 130–170 Å diameter particles. (3) A surface with fairly closely packed 125–135 Å diameter particles. (4) A surface with very closely packed 90 Å diameter particles. When transitions from one type surface to another occur with no intervening ledge representing a half-membrane, they are always of two sorts: (*a*) Type one merging into a type two. (*b*) Type three merging into a type four. We then reason that such regions of transition occur in areas where membranes change from a stacked to an unstacked state or vice versa. This is admittedly speculative, but the model that results from this speculation appears to explain all observed fracture faces, as will presently be seen. This interpretation calls face one an unstacked region and face two a stacked region of the same lamella. In the same way, face four would be a region of unstacked lamella, while face three would be a stacked region. Transitions of both these sorts may be seen in Figs. 5 and 6.

To obtain further evidence regarding this stacking hypothesis and to determine positively which fracture surfaces face each other in the intact membrane, we employed the double-recovery technique, so that complementary halves of the fractured chloroplast membranes might be examined. Reference to Figs. 7 and 8 and to Figs. 9 and 10, both matching pairs, makes this clear.

We have departed from the usual practice of presenting the matching pairs as mirror images, and instead have inverted one negative in each pair to produce prints with complementary sur-

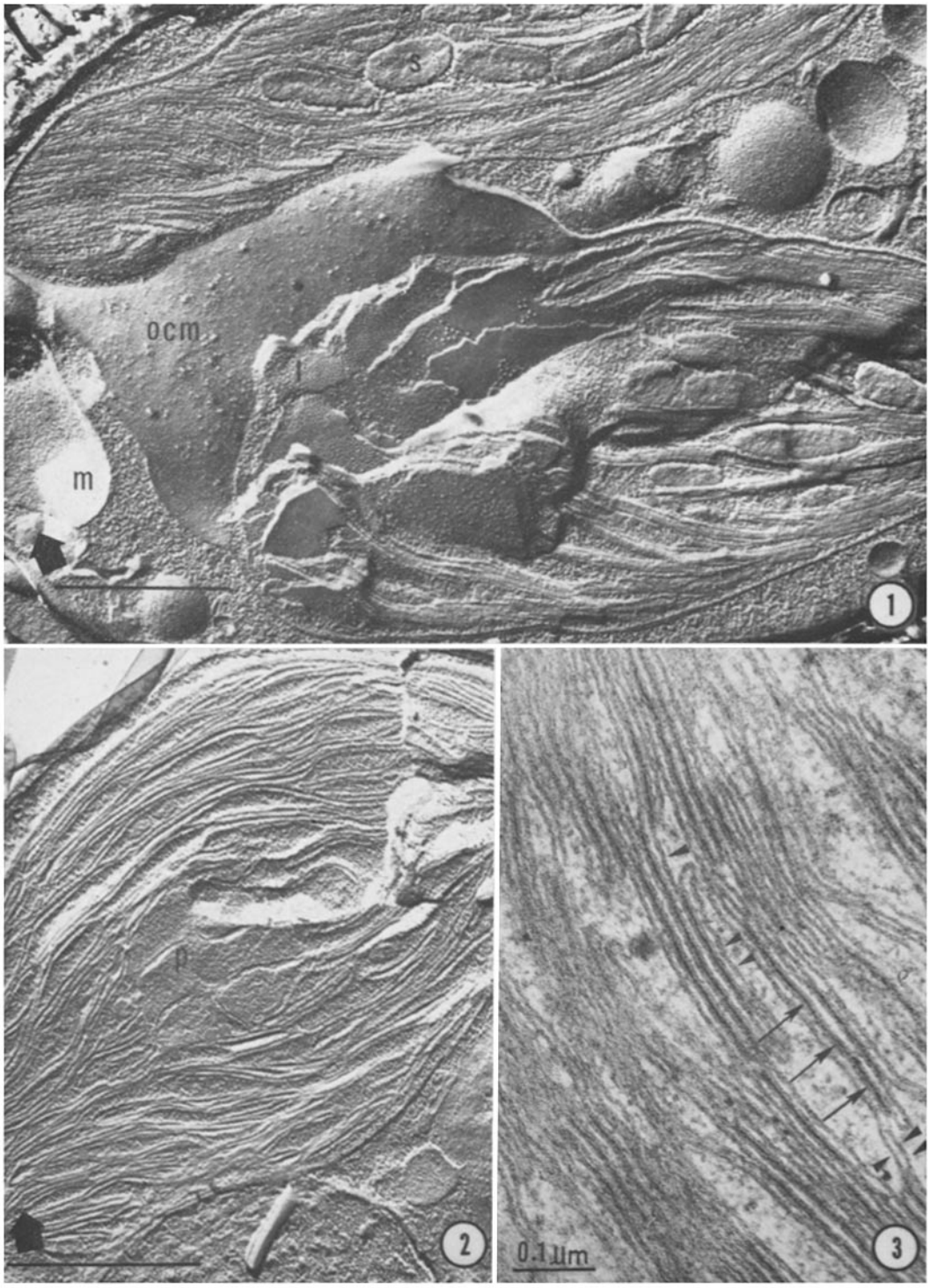


FIGURE 1 Freeze-etched *O. marssonii* cell showing mitochondrion (*m*) at left, a stretch of outer chloroplast membrane (*ocm*), the cup-shaped chloroplast, starch grains (*s*), and surface views of lamellae (*l*). Large arrow indicates direction of platinum shadow. × 26,400.

FIGURE 2 Cross-fracture of *Oocystis* chloroplast showing lamellae extending the length of the chloroplast and associating in stacks of three to five. Pyrenoid (*p*) is also shown. Large arrow indicates direction of platinum shadow. × 29,400.

FIGURE 3 Chloroplast lamellae showing details of membrane associations. Entire arrows outline areas of stacked membrane; arrowheads mark unstacked membrane regions. × 126,500.

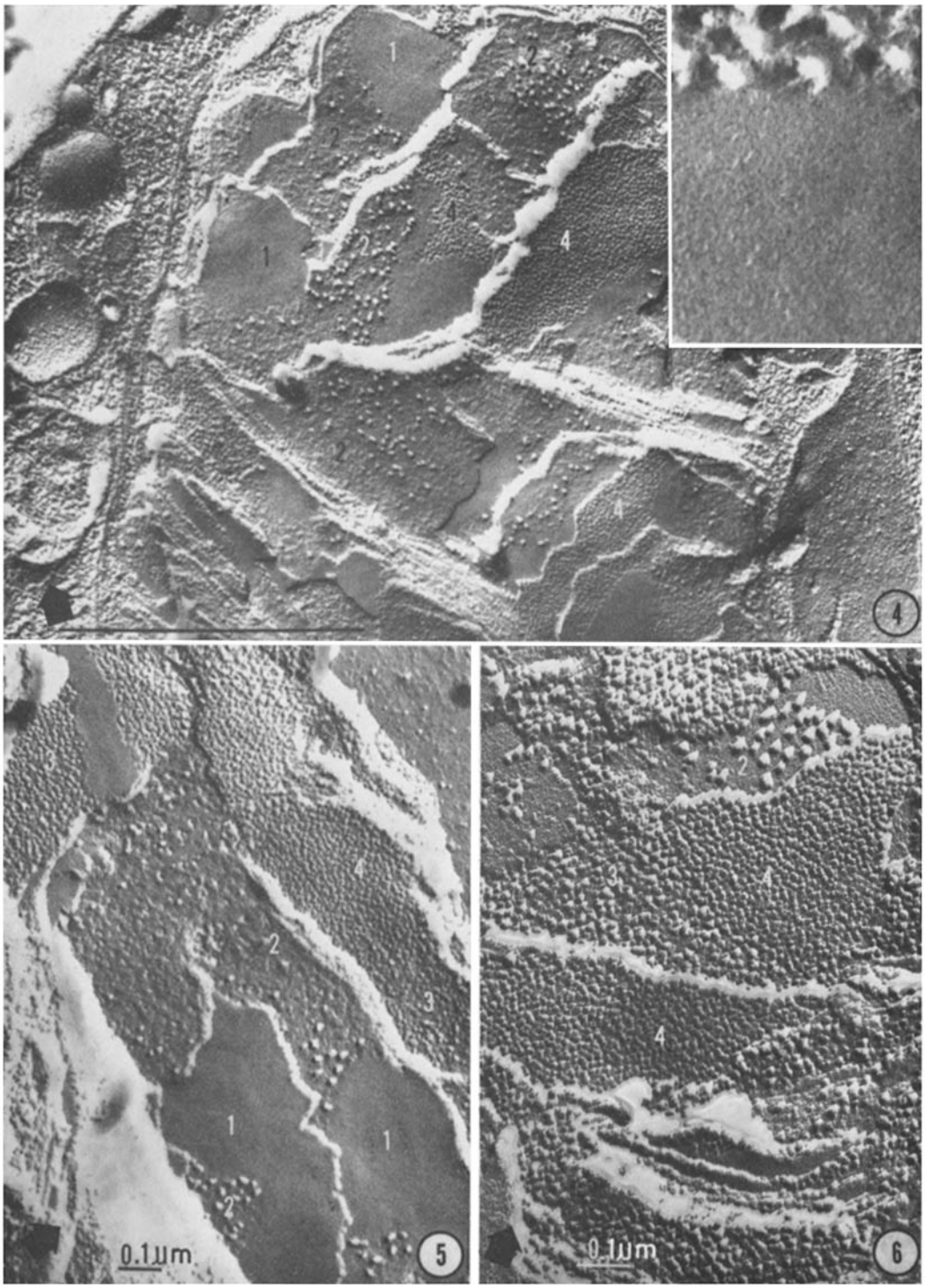
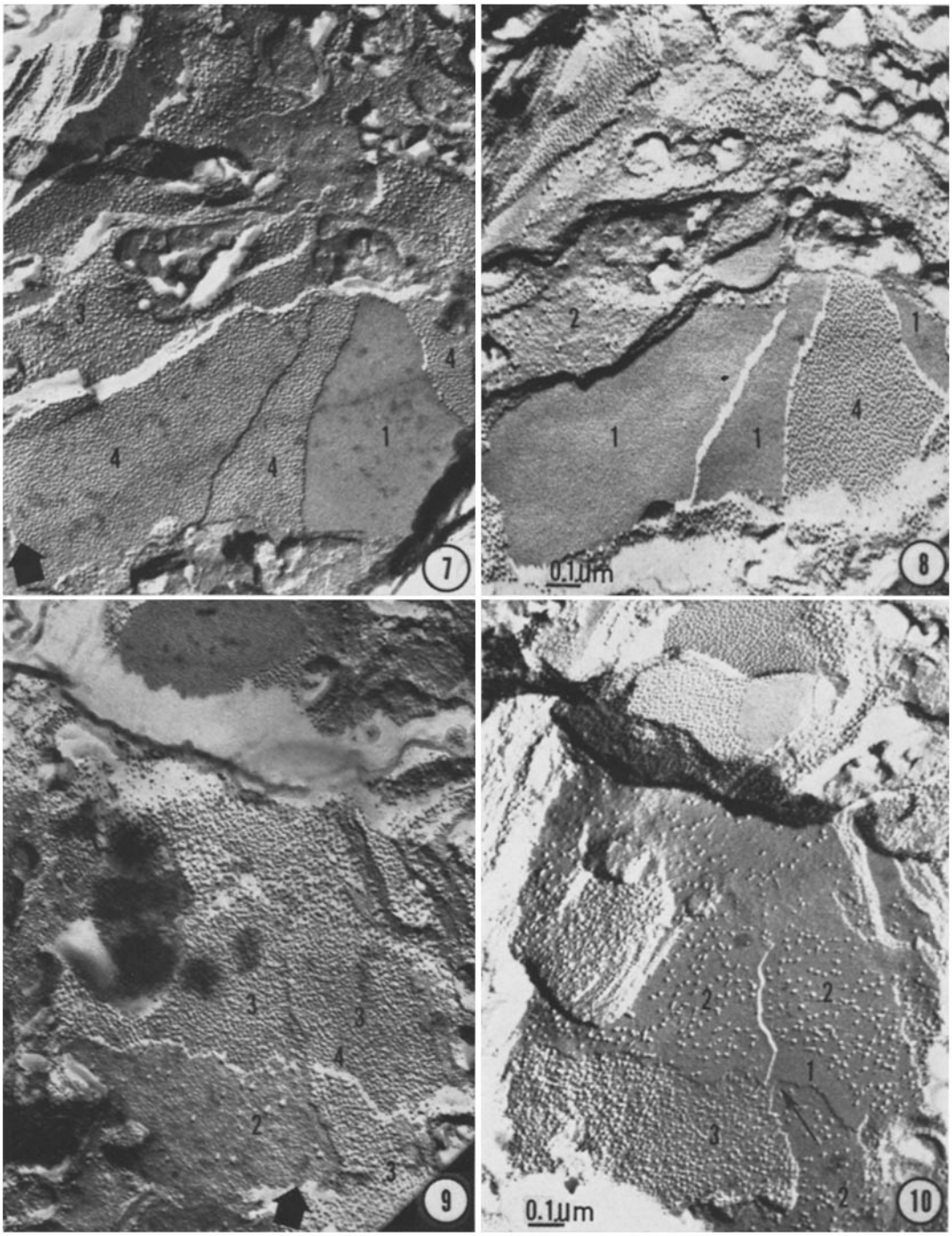


FIGURE 4 Region of freeze-etched chloroplast. Surfaces one, two, and four described in text may be seen. × 46,000. *Inset* shows a type one face at high magnification; this intergrades into a type two face. × 286,000.

FIGURE 5 Higher magnification of chloroplast showing all four fracture face types. × 62,700.

FIGURE 6 Slightly oblique fracture showing how fracture faces alternate across a stack of lamellae. A type four face intergrades into a type three face near center. × 74,000.



FIGURES 7 and 8 Matching fracture faces from double-recovery procedure, printed so that corresponding structures lie similarly oriented in both prints. It may be seen that surface type one faces type four in the intact membrane. Large arrow indicates direction of platinum shadow. × 49,400.

FIGURES 9 and 10 Double-recovery pair showing that the type two surface faces the type three surface. Large arrow indicates direction of platinum shadow. × 50,600.

faces in the same areas in both cases. We find such an arrangement easier to interpret.

Surface one always faces surface four, and surface two always faces surface three. We interpret the smooth surface one as a lipoidal half-membrane, the other half of this membrane being composed of the 90 Å diameter particles, probably in a lipoidal matrix.

Having been made aware of the dangers of a simple particle size averaging by the work of Goodenough and Staehelin (5), we plotted the particle sizes present on the various surfaces in the same way these authors did. The type one surface is of course devoid of particles. The type two surface contains particles ranging from 100 to 180 Å. The majority of the particles present, however, fall into two distinct peaks at 130 and 170 Å. Type three surfaces have particles in the range 100–135 Å, with a single peak at 125 Å. Type four surface particles range from 70 to 120 Å in size, with a single peak at 90 Å. Like Goodenough and Staehelin, we admit some difficulty in reaching reliable size figures for the closely packed particles on the types three and four surfaces.

Although we lack the elegant evidence from studies of mutant cell lines presented by Goodenough and Staehelin (5), applying to our system their conclusion that the largest particles in the *Chlamydomonas* chloroplast lamellae are shared between adjacent membranes of stacked lamellae, we can explain all our observations. We suggest that the fracture plane in such a pair of stacked membranes can pass between the nonpolar ends of the lipids of either membrane in the stacked pair. In fact, Figs. 9 and 10 illustrate just such a case, in which the fracture plane has alternated between the center layers of two adjacent membranes. A type two surface may be seen adjacent to a type three surface, separated by the sort of ledge one would expect from two half-membranes (Fig. 10). A type two surface would arise where one and one-half membranes fractured away in a stacked region, leaving a half-membrane with a few scattered 130–170 Å particles, most of the shared particles having been pulled away by the fracture and leaving the large depressions evident between the scattered particles (Figs. 5, 9, 10). Conversely, when only one half-membrane fractures away, many of the shared particles stay with the remaining one and one-half membranes, their less exposed upper portions creating the closely packed 125 Å images of the type three surface. Thus we are suggesting that the particles on the type two and type three surfaces are the same shared particles which are exposed to a greater or lesser extent by the fracture plane. Our double-recovery results seem to bear this out.

A diagram clarifying these statements is presented as Fig. 11. It will be seen that there are two sizes of particles illustrated, 90 and 170 Å in diameter. The 125 Å particles are in every case the matching face to the latter. Variation in size of particles on the type two surface is attributed to varying amounts of the particles themselves being ripped away during the fracture. Fracture planes are illustrated to correspond to the types we have found and upon which the proposed model is based. Membranes not tightly apposed to adjacent membranes by stacking of the lamellae, that is, unstacked regions, would yield only two sorts of fracture faces, types one and four, containing only particles in the 90 Å size range and these only in one half of the membrane. Larger particles would be found in stacked regions, and would appear as closely packed 125 Å particles or scattered 130–170 Å ones, depending on which membrane in the stacked pair was fractured.

DISCUSSION

Studies which are sufficiently complete to allow comparison with the present one include those of Park and Pfeifhofer (13), Branton and Park (4), Arntzen et al. (1), and Goodenough and Staehelin (5). The first three are in essential agreement among themselves and all deal with the grana regions of higher plants. They show two sizes of particles, 110 and 175 Å, present there. Working with the filamentous green alga *Chaetomorpha*, Robinson (15) has recently shown chloroplast lamellae similar to those of *Oocystis*. Although the particle sizes do not correspond exactly, we find Robinson's face D equivalent to our type one, his face A equivalent to our type four, his face B equivalent to our type two, and his face C equivalent to our type three. Robinson's face B apparently has more particles and fewer depressions than our type two, but this might be due to preparation procedures or differences in replica quality. Goodenough and Staehelin (5) stress the continuum of particle sizes observed on *Chlamydomonas* lamellae, and our results indicate that a similar range of sizes exists on *Oocystis* lamellae. They assume that *Chlamydomonas* chloroplasts will be similar structurally to grana regions of higher plants and find 105 and 160 Å size peaks corresponding to similar particle sizes in higher plant

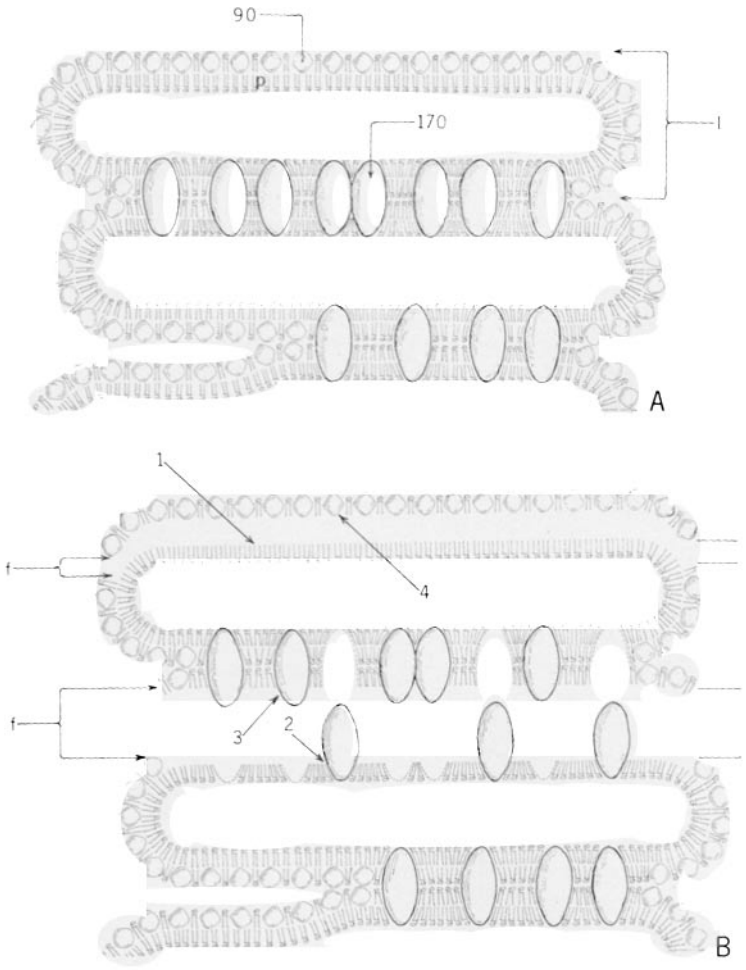


FIGURE 11 Drawing A represents our conception of the arrangement of the components we have seen in the *Oocystis* chloroplast lamellae. Drawing B illustrates the possible ways such a membrane could fracture (*f*) to create the types one, two, three, and four fracture surfaces. Top lamella is completely unstacked along its upper surface and entirely stacked along its lower. The middle lamella is partly stacked and partly unstacked along its lower surface. *p*, Polar lipid; *l*, lamella. 90 and 170 refer to sizes of particles in Angstroms.

grana. Furthermore, they also describe a 130 Å size peak on the same face as that bearing the 160 Å peak. Close comparison of these results with ours convinces us that there are basic differences explainable only by presuming major differences in the two organisms. The type Bu face in *Chlamydomonas* appears identical to the *Oocystis* type two face, except that the 130–170 Å particles we find in *Oocystis* are somewhat larger than the predominant 135 Å particles on this face in *Chlamydomonas*. Both algae have obvious depressions on this face. Facing this surface in *Chlamydomonas* is the Cu face with particles ranging from 75 to 155 Å in diameter, similar to the type three face in *Oocystis*. We suggest, however, that these complementary faces in *Oocystis* evidently represent stacked regions of the lamellae, with all visible particles shared between apposed membranes. Goodenough and Staehelin (5) found these surfaces in unstacked regions. *Oocystis* has no surface equivalent to the Bs face in *Chlamydomonas*, although we do believe our type four face equivalent to the Cs face of *Chlamydomonas*. Since the *Oocystis* type four faces the smooth type one, we must conclude that these faces occur in an unstacked area. The striking correspondence in particle size

classes between our type two face and the *Chlamydomonas* Bs face cannot be ignored. Admittedly we find very rare areas on our type two face that approach *Chlamydomonas* Bs faces in particle frequency. But such areas are so infrequent in relation to the large expanses of stacked lamellae seen in cross fracture and in thin section that we cannot conceive a consistent face of this type as comprising a stacked region in *Oocystis*.

In efforts to explain the surprising differences between *Chlamydomonas* and *Oocystis*, both members of the division Chlorophyta, the green algae, we have freeze-etched whole cells of *C. reinhardtii*. Although preservation is not ideal, we see surfaces identical with those described by Goodenough and Staehelin. This would seem to eliminate difficulties in our procedures. We are unable to find physiological data on *Oocystis* and so have attempted to extract the chlorophylls of the alga with 80% acetone for spectrophotometric characterization. So far the cells have resisted our efforts at this extraction and at cell breakage. Procedures of extraction and disruption effective with *Chlamydomonas* and *Euglena* have no effect on *Oocystis*.

In view of these difficulties, we must attribute the differences between our results and those of Goodenough and Staehelin (5) to basic differences in the algae involved until additional information becomes available. Our results also illustrate the danger of wholesale and automatic extrapolation of results obtained with one organism to generalizations about cells in general or even about organisms in the same taxonomic group.

The fluid mosaic membrane model proposed by Singer and Nicholson (16) readily lends itself to slight modifications to accommodate our data. The matching type one and type four surfaces described here seem to demand an asymmetric membrane with a high percentage of particulate material in one half and none in the other. Should the obvious possibility that one half-membrane is largely protein and the other largely lipid prove true, this would be a unique situation that would have to be considered in any model. Such asymmetric areas are in fact present in the Singer-Nicholson illustration. The absence of any depressions on the smooth type one surface corresponding to the 90 Å particles on the complementary surface also requires some attempt at explanation. If one assumes a Singer-Nicholson membrane such that one-half of the bilayer is composed entirely of polar lipids, with no penetration of this layer by the particulate component of the opposing half of the bilayer, and stipulates that the particles are interspersed among polar lipids, there is no reason to expect that the type one face would exhibit depressions. Such an asymmetric membrane does not appear to be inconsistent with the fluid mosaic model as recently set forth; consequently we offer this as the best explanation for an admittedly puzzling observation. Also, it is possible that shallow depressions in the type one face might have been obliterated by plastic deformations in the membrane during fracture. We agree with Goodenough and Staehelin that the 130–170 Å particles are shared between two adjacent unit membranes, although we lack the elegant direct evidence in *Oocystis* that they obtained in *Chlamydomonas*. However, we find no real evidence of smaller particles interspersed among the 175 Å ones in *Oocystis* and prefer instead to consider that the stacked areas may contain only a single kind of particle which is exposed to a greater or lesser extent by the randomness of the fracture plane.

Obvious unanswered questions include the appearance of the unfractured, deep-etched thylakoid membrane, the functions of the various particles described here, and the intriguing possibility that the 170 Å particles might be made of the 90 Å particles as subunits. Approaches to solution of these problems are in progress and information thus obtained will hopefully further confirm and add details to the tentative model presented in Fig. 11.

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