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CHLOROPLAST MEMBRANE STRUCTURE

INTRAMEMBRANOUS PARTICLES OF DIFFERENT SIZES MAKE CONTACT IN STACKED MEMBRANE REGIONS

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SUMMARY

The supramolecular architecture of stacked thylakoid membrane regions of class II spinach chloroplasts has been investigated by means of freeze-fracture electron microscopy. Such membranes contain two basic types of intramembranous particles: large particles, which are found on the fracture face of the lumenal membrane leaflet (Bs face), and smaller ones which are found on the fracture face of the external leaflet (Cs face). By analyzing thylakoid membranes containing geometrical arrangements of intramembranous particles it is shown (a) that within the plane of each membrane approximately two small particles are associated with each large particle, and (b) that normal thylakoid stacking involves the connection of large particles of one membrane to small particles of the other and vice versa.

If the two types of particles are related to Photosystems I and II, as suggested by circumstantial evidence, then our observations provide support for the idea that maximum Photosystem I–Photosystem II interaction is obtained by intermembrane subunit interaction in grana stacks. To this end, our results suggest that stacking should enhance the quantum yield at very low light intensities.

INTRODUCTION

Chloroplast membranes possess the capacity to "fuse" together over long distances, forming grana in higher plants and stacks in green algae. The significance of these structures is still uncertain but their widespread distribution implies that they convey a selective advantage to the bearers of this property. In recent years biochemical, biophysical and physiological studies have revealed that stacked thylakoid membranes possess a variety of unique properties [1–13], most of which seem to be explainable by the theory that membrane fusion improves the efficiency of energy coupling between Photosystems II and I [9, 10].

Biochemical fractionation studies of thylakoid membranes separated into grana and stroma membrane regions [1, 14, 15] have shown that stacked membranes differ in their composition (chlorophyll *a*/chlorophyll *b* ratio, and Photosystem II and Photosystem I content) from unstacked ones, thus indicating that thylakoid membrane stacking involves more than a non-specific adhesion between adjacent membrane surfaces.

These differences in membrane composition are matched by differences in the freeze-cleave morphology of stacked and unstacked thylakoids. In 1971 Goodenough and Staehelin [16] demonstrated that in freeze-cleave replicas stacked membrane regions of the green alga *Chlamydomonas reinhardi* may be distinguished from unstacked regions by the presence of arrays of large particles on their B face (fracture face of the lumenal membrane leaflet); unstacked stroma membrane regions possess only few such particles. Morphological differences related to stacked and unstacked membrane regions were also observed on the complementary C fracture faces, although the differences did not appear as pronounced because of the overall denser packing of the C face particles. Particle size histograms revealed furthermore that the average C face particle was considerably smaller (80–100 Å) than the average B face particle (120–140 Å). Since most of the large B face particles were concentrated in the stacked membrane regions, it was proposed that they may be involved in the stacking process, thus they were termed stacking particles. To emphasize that point a chloroplast membrane model was proposed showing the stacking particles of adjacent membranes making head-to-head contact in the stacked membrane regions. The chloroplast membrane model of Pendland and Aldrich [17] goes even one step further, depicting the stacked membrane regions being held together by large rod-shaped particles that are shared by the two membranes. Both models exhibit a symmetrical distribution of intramembranous particles as found in most intercellular junctions [18]. In neither paper were the two other possible configurations either evaluated or considered, namely, that no special association between particles of adjacent stacked membranes existed, or that in stacked membrane regions the connections between adjacent thylakoids may be asymmetrical, i.e. that the large particles of one membrane might connect to small particles in the other and vice versa.

Since the particles visualized on freeze-cleaved thylakoid membrane faces could very well correspond to macro-molecular complexes of Photosystem II and Photosystem I components (although to date no positive correlation of this type has been achieved), a knowledge of whether particles link up in stacked membrane regions, and if they do so whether similar or dissimilar particles make contact, might help elucidate how stacked membranes are held together, and what the significance of this intriguing membrane phenomenon may be.

Using thylakoid membranes containing geometrical arrangement of intramembranous particles we demonstrate that dissimilar particles link up in stacked regions of thylakoid membranes.

MATERIALS AND METHODS

Spinach leaves of the Bloomsdale Savoy variety were obtained commercially. 300 g of deveined and water-washed leaves were homogenized for 30 s in a Waring blender containing 500 ml either of 0.05 M sodium phosphate buffer (pH 7.3), 0.35 M NaCl, 0.001 M EDTA or 0.05 M potassium phosphate buffer (pH 7.2), 0.3 M sucrose, 0.01 M KCl. The homogenate was passed through eight layers of cheesecloth and centrifuged for 3 min at 300×g. The pellet was discarded and the supernatant was centrifuged for 10 min at 2000×g. The crude preparation of class II plastids was resuspended in 50–100 ml of isolation buffer and divided into aliquots for further processing into freeze-etch specimens of the following types: (a) chloroplasts pelleted and frozen in isolation buffer without further treatment; (b) chloroplasts infiltrated with 30 % glycerol for 30 min, pelleted and frozen on copper discs; (c) chloroplasts fixed in 0.5 or 1 % glutaraldehyde for 10 min, pelleted, resuspended in fresh isolation buffer, infiltrated with 30 % glycerol for 30 min, pelleted and frozen. Freezing of the pelleted chloroplasts, freeze-cleaving of the frozen specimens and cleaning of the replicas was carried out as described by Goodenough and Staehelin [16].

RESULTS

Freeze-cleaved thylakoids of isolated, class II spinach chloroplasts exhibit four fracture faces (Fig. 1), generally referred to as Bs (stacked), Bu (unstacked), Cs and Cu faces [16]. Bs and Bu represent fracture faces of the lumenal leaflet of thylakoid membranes, Cs and Cu fracture faces of the external leaflet. The faces Bs and Cs represent complementary type images of stacked membrane regions, and Bu and Cu corresponding views of unstacked membrane areas. Bs faces are most readily recognized because of their characteristic compliment of large particles. (As demonstrated by particle size histograms [16] the "average" Bs face particle has a diameter of approx. 130 Å, but the spread of particle sizes is such that nearly one-third have diameters ranging from 150 to 180 Å. Since the large particles seem to be composed of subunits (refs 16, 19 and 20, and Staehelin, unpublished observations), the large differences in particle sizes could result from the pulling apart of subunits during the fracturing process.) The complementary Cs faces (Fig. 2) carry numerous small particles (average diameter 80 Å) located between large pits produced by the tearing away of the large Bs face particles. From these images we can deduce that stacked thylakoids contain both large and small intramembranous particles, the small particles being approximately twice as numerous as the large ones. In most chloroplasts both types of particles are randomly distributed (Figs 1 and 2), and in such specimens it is impossible to determine (a) whether any special spacial relationship exists between large and small particles within the plane of each membrane, and (b) whether particles in adjacent stacked membranes line up in any specific way.

Answers to these questions can, however, be obtained if the particles in a stacked region become organized into a geometrical array. For such membrane areas testable models of membrane structure can be developed that not only demonstrate that particles in adjacent membranes must be specifically connected to each other, but also allow us to determine whether equally or unequally sized particles of adjacent membranes link up.

As first reported by Park and co-workers [19, 20] geometrical arrangements of thylakoid membrane particles can occasionally be observed in preparations of isolated chloroplasts. Analysis of numerous micrographs of freeze-cleaved thylakoid membranes possessing geometrical arrangements of intramembranous particles has revealed the following specific structural associations between the large and small particles of stacked membrane regions: Figs 3, 4 and 5 depict small Cs face areas containing small particles in double rows separated by rows of closely spaced large

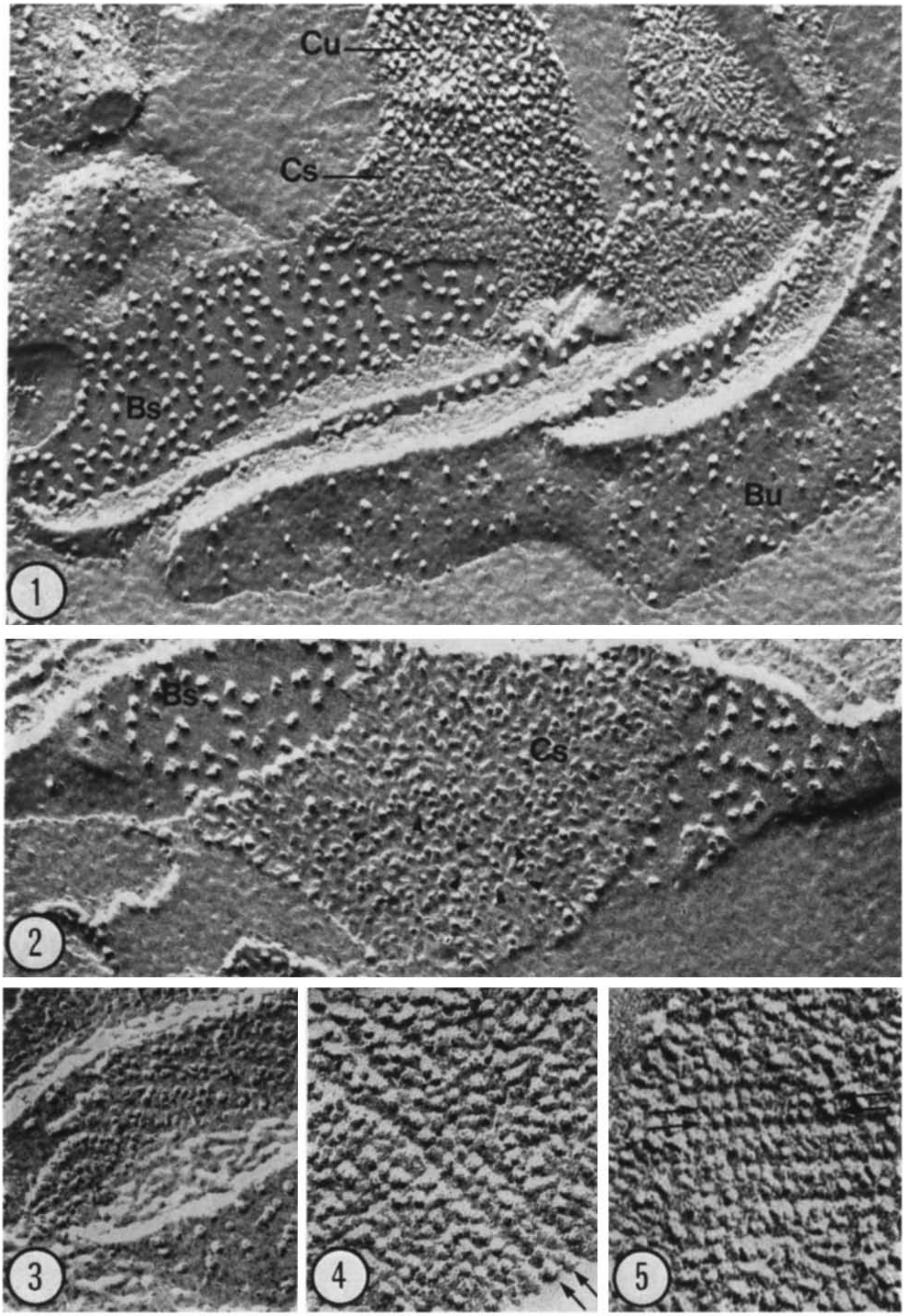
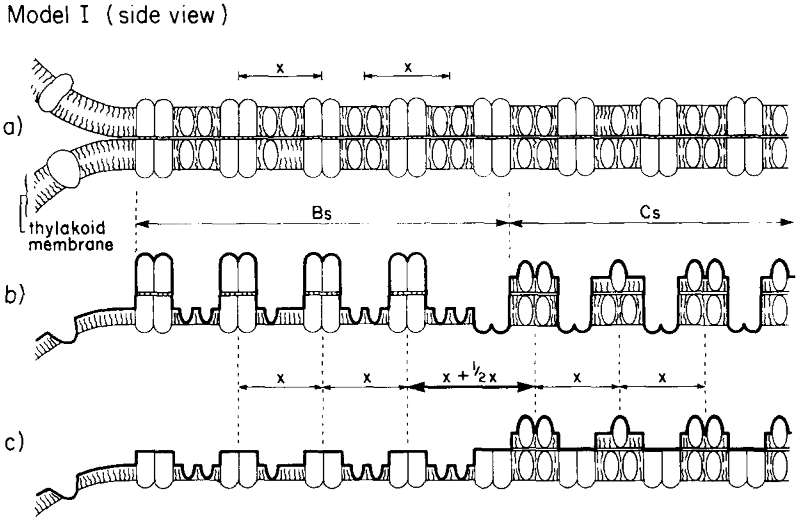


Fig. 1. Freeze-cleaved isolated thylakoids of spinach exhibiting the four fracture faces designated Bs, Bu, Cs, and Cu by Goodenough and Staehelin [16]. Bs and Cs are complementary-type membrane faces of stacked membrane regions, while the complementary-type faces Bu and Cu are



Model I (side view)

Fig. 6. (a) Model I of two stacked thylakoid membranes containing rows of regularly spaced large particles making head-to-head contact. The smaller particles are confined to the spaces in between the rows of large particles. (b and c) Diagrammatic representation of two possible cleaving patterns and resulting fracture faces (Bs and Cs) through the stacked membrane region shown in a (for details see text). As indicated by the spacing markers "X" and "$X+\frac{1}{2}X$" these different fracturing modes do not affect the spacing of rows of Bs face particles and of Cs face ridges. This model predicts that as the cleavage plane passes from the lower Bs to the upper Cs face the spacing between the last Bs particle row and the first Cs ridge (containing the small particles) would be "$X+\frac{1}{2}X$", while the spacing of the adjacent particle rows and ridges would be "X". An alternate model is illustrated in Fig. 7.

pits or grooves. The double rows of small particles are difficult to resolve clearly; in most instances they appear fused into a single broad ridge as seen in Fig. 8. Careful examination of such arrays of small Cs face particles suggests that each large particle may associate laterally with two smaller particles. This type of association is also supported by particle density measurements, which reveal that Cs faces contain nearly twice as many particles (approx. 3600 μm^{-2}) as Bs faces (approx. 2000 μm^{-2}).

Figs 6, 7, 8 and 9 relate to the association of particles in adjoining, stacked thylakoid membranes. The presence of a special spacial relationship between particles

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associated with unstacked membrane areas. Note the characteristic compliment of large particles on the Bs faces. Magnification, ×95 000.

Fig. 2. Higher magnification of a typical Cs face. Between the large pits (arrowheads), formed by the removal of the large Bs face particles, numerous smaller particles may be recognized. The random distribution of Bs and Cs face particles as illustrated in this micrograph and in Fig. 1 does not permit the detection of any special spacial relationship between the two types of particles. Magnification, ×132 000.

Fig. 3. Cs face with particles organized into rows. Careful examination of this micrograph as well as of Figs 4 and 5 indicates that the particles are arranged in double rows separated by deep grooves formed by rows of closely spaced holes. Magnification, ×125 000.

Figs 4 and 5. Cs faces exhibiting small particles arranged in double rows (arrows) separated by single rows of closely spaced depressions (grooves). Magnification, ×205 000.

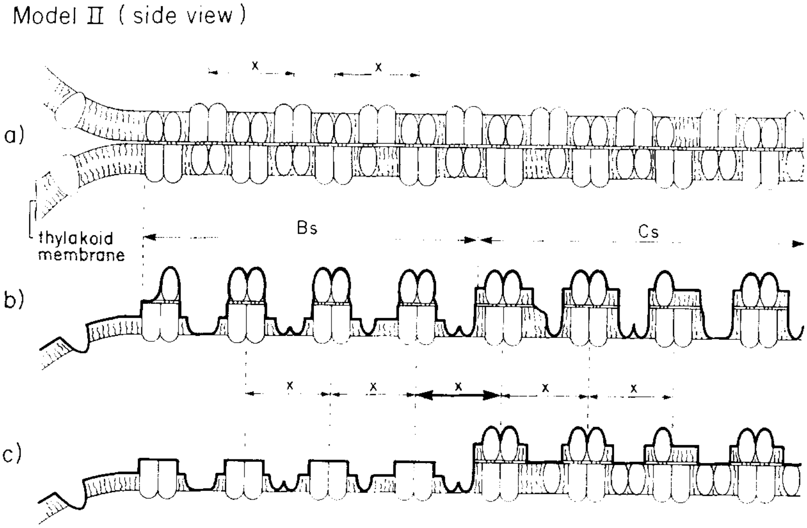
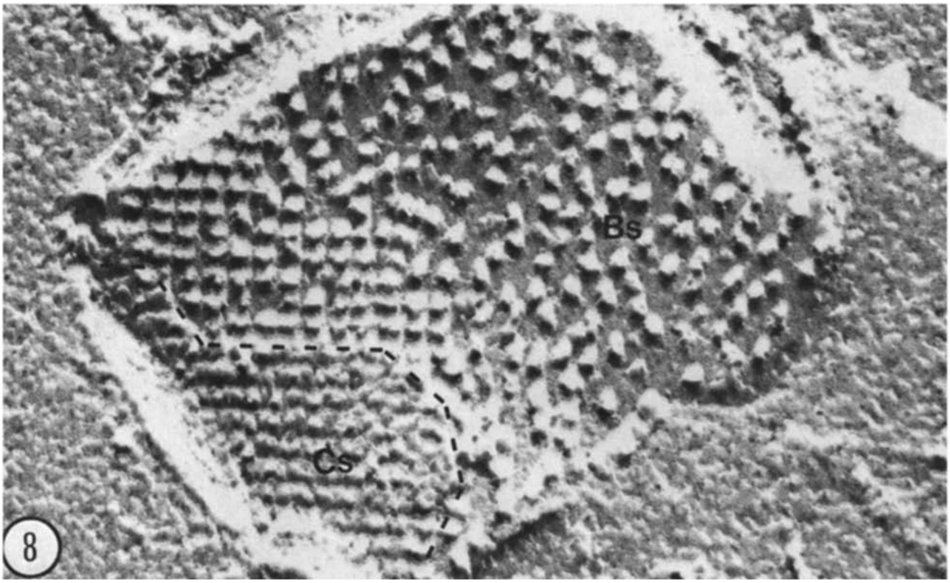


Fig. 7. (a) Model II of two stacked thylakoid membranes containing rows of evenly spaced large and small particles. In contrast to Model I, the large particles of one membrane link up with the small particles of the adjacent membrane. (b and c) Diagrammatic representation of two possible cleaving patterns and resulting fracture faces (Bs and Cs) through the stacked membranes shown in a. Diagrams b and c differ only in the way in which the associated particles of adjacent membranes are cleaved (see the text for further details). These different fracturing modes do not interfere with the measurable spacings between the rows of Bs face particles and the Cs face ridges. This model predicts that as the cleavage plane passes from the lower Bs to the upper Cs face the spacing between the last Bs particle row and the first Cs ridge would be "*X*", i.e. the same as the spacing between the adjacent rows of particles and ridges. As demonstrated in Figs 8 and 9, stacked thylakoid membranes appear to conform to Model II.

of adjacent membranes can be deduced from the fact that whenever particle arrays of stacked membrane regions are cleaved as to reveal adjacent Bs and Cs faces as illustrated in Fig. 8, a perfect alignment of particle rows on both faces can be observed. If the adjacent membranes would stick together in a non-specific way, then one would expect to find at least occasionally a lack of alignment between particle rows on adjoining Bs and Cs faces, or even a random array of particles on one face connecting to a geometrical array of particles on the other. We have found no such deviations in any of the numerous micrographs we have examined to date.

Having established that particles of adjacent membranes become aligned with each other upon stacking, we may address the question whether equally or unequally sized particles connect to each other. Fig. 6a illustrates Model I of stacked thylakoid membranes, which corresponds basically to the models of Goodenough and Staehelin [16] and Pendland and Aldrich [17]. According to this model the regularly spaced large particles (repeating distance "*X*") make head-to-head contact, while the smaller particles are confined to the spaces in between the rows of large particles (each large particle symbolizes a particle row perpendicular to the plane of the page). Figs 6b and 6c show the fracture faces produced by two possible modes of cleaving through the stacked membrane region shown in Fig. 6a. Diagrams b and c differ in the way in which the large contacting particles are cleaved, reflecting on the present uncertainty



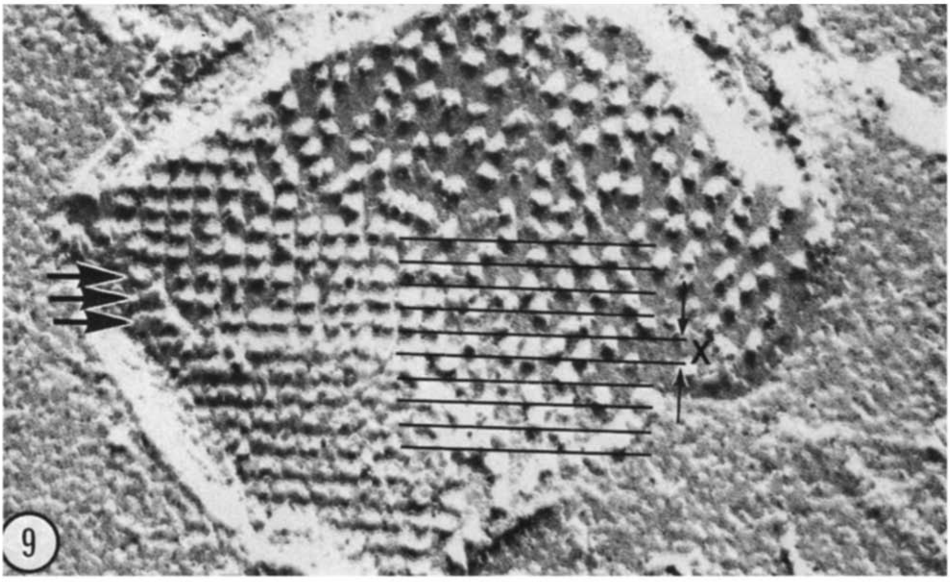


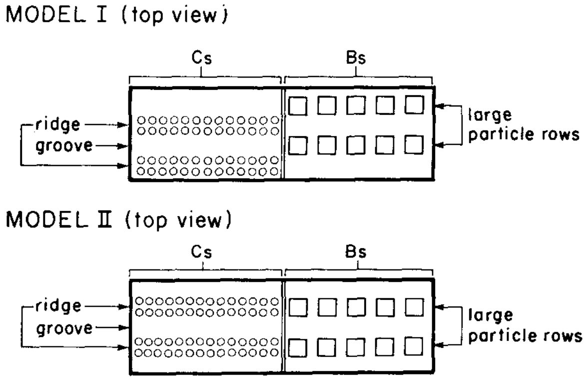
Fig. 8. Freeze-cleaved stacked thylakoid membranes of an isolated spinach chloroplast. On the right side the particles on the Bs face are randomly distributed, while on the left side they are organized into a square lattice. This lattice can be traced without interruption into the overlying Cs face, the edge of which is marked by a dashed line. Magnification, ×165 000.

Fig. 9. The same micrograph as shown in Fig. 8, but marked to show the spacing of the rows of Bs face particles and of Cs face ridges. Where the fracture plane jumps from the lower Bs to the upper Cs face the spacing between the last row of particles and the first ridge is "*X*", i.e. the spacing is the same as between adjacent rows of particles, respectively ridges. Thus the cleaving pattern corresponds to the prediction made in Model II illustrated in Fig. 7. The arrows point to short segments of Cs face ridges forming extensions of Bs face particle rows, again confirming Model II as pointed out in Fig. 10. Magnification, ×165 000.

in interpretation of particle heights of freeze-cleaved membrane faces. (As demonstrated by Goodenough and Staehelin [16] the tips of Bs face particles frequently appear to be approximately level with those of adjacent Cs face particles. This finding can be explained by the cleaving pattern shown in Fig. 6b. It could also be explained by the pattern shown in Fig. 6c, if the particles become plastically deformed and elongated when they are pulled apart. Since plastic deformation of membrane components occurs during freeze-cleaving even at the temperature of liquid helium (4 °K) [21], plastic deformation of the large particles may well account for their unusual height.) Fortunately, however, these different fracturing modes do not affect the measurable spacings "X" and "$X+\frac{1}{2}X$" between the rows of Bs face particles and the ridges on Cs faces, which are essential for testing the different models. This model predicts that as the cleavage plane passes from the lower Bs to the upper Cs face, the spacing between the last Bs particle row and the first Cs ridge (containing the small particles) would be "$X+\frac{1}{2}X$", while the spacing of the adjacent particle rows and ridges would be "X".

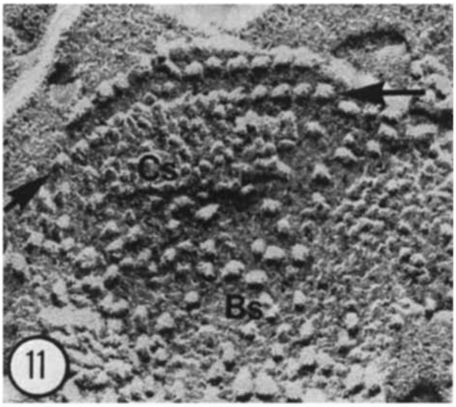
Fig. 7a depicts an alternate model to Fig. 6a. This Model II of stacked thylakoid membranes also contains rows of evenly spaced large and small particles (repeating distance "X"). However, in contrast to Model I, the large particles of one membrane link up with the small particles of the adjacent membrane. Figs 7b and 7c illustrate the two fracturing modes discussed for Figs 6b and 6c and the expected spacings between the Bs particle rows and Cs ridges. Note that the spacing between the last row of Bs particles and the first Cs ridge is "X" and not "$X+\frac{1}{2}X$" as in Model I.

Fig. 8 demonstrates a stacked thylakoid membrane region of a freeze-cleaved spinach chloroplast. On the right side of the exposed Bs face the particles are randomly distributed, while on the left side they are organized into a square lattice. In the middle of the lattice region the fracture plane jumps from the lower Bs face to the


MODEL I (top view)

MODEL II (top view)

Fig. 10. Schematic diagrams illustrating in top views the relationship of the Cs face ridges and grooves to the Bs face particle rows as predicted by Model I (Fig. 6a) and by Model II (Fig. 7a). Note that in Model I the rows of Bs face particles line up with the Cs face grooves located between the ridges. In contrast, Model II predicts that the Bs face particle rows line up with the Cs face ridges, and that the Cs face grooves coincide with the spaces in between the Bs face particle rows. The images of stacked thylakoid membranes shown in Figs 9, 11 and 12 seem to correspond to Model II.



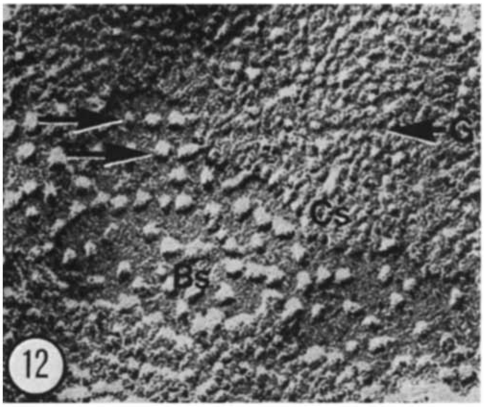


Fig. 11. Freeze-cleaved stacked thylakoid membranes depicting both Bs and Cs fracture faces. Note the continuity of the row of Bs face particles marked by arrows with the ridge material at the edge of the Cs fracture face. This arrangement corresponds to the image predicted for Model II in Fig. 10. Magnification, ×165 000.

Fig. 12. Bs and Cs fracture faces of a spinach chloroplast. In accordance with Model II (Fig. 10) the groove (G) on the Cs face is lined up with the space between the two Bs particle rows marked with arrows. Magnification, ×165 000.

upper Cs face as illustrated in Figs 6b, 6c, 7b and 7c. Fig. 9 shows the same micrograph as Fig. 8, but marked with lines to indicate the spacing of the rows of Bs particles and Cs ridges (compare with Figs 6a and 7a). Where the fracture plane jumps from the Bs to the Cs face the spacing between the last row of Bs particles and the first Cs ridge is "X", i.e. the same as between adjacent rows of particles and adjacent ridges. Thus, the cleaving pattern corresponds to the prediction made in Model II (Fig. 7a), suggesting that in stacked thylakoid membrane regions the large particles of one membrane connect to the small particles in the other.

This interaction is confirmed further by images of stacked thylakoid membranes in which the Bs face particle rows are seen as extensions of Cs ridges. Diagram Fig. 10 illustrates in top views the relationship between Cs face ridges and grooves and Bs particle rows as predicted by Model I (Fig. 6a) and by Model II (Fig. 7a). Figs 9, 11 and 12 all show arrangements of particles, ridges and grooves that conform to Model II in Fig. 10. Thus, all our replica images seem to support the theory that large and small intramembranous particles of adjacent thylakoid membranes link up in stacked membrane regions.

DISCUSSION

It is now generally accepted that the intramembranous particles exposed on the fracture faces of freeze-cleaved biological membranes correspond to the integral membrane proteins of the fluid mosaic membrane model [22–25]. For this reason freeze-cleaving and freeze-etching techniques have become important tools for investigating the distribution and properties of integral membrane proteins in a variety of membrane systems.

As shown by several laboratories [16, 26–28] and confirmed in the present communication (Figs 1 and 2), freeze-fractured thylakoids reveal the presence of two basic types of intramembranous particles in stacked membrane regions. These particles differ both in their average size and in their cleaving behavior, the smaller ones cleaving with the Cs face (Fig. 2), and the larger ones with the complementary Bs face (Figs 1 and 2).

Biochemical studies have demonstrated that it is possible to fractionate thylakoid membranes into grana and stroma membrane regions by differential centrifugation of class II chloroplasts following breakage with digitonin [1, 29–31] or with a French pressure cell [14, 15]. Testing of the fractions revealed that stroma membranes contain virtually only Photosystem I activity, while grana membranes possess both Photosystem I and Photosystem II activities in similar amounts. In light of these biochemical observations it is tempting to speculate that the two types of intramembranous particles of stacked thylakoid membrane regions might be related to Photosystem I and Photosystem II complexes. The small particles that cleave with the Cs face could correspond to one photosystem and the large Bs face particles to the other. Which system might correspond to which type of particle is still unknown, but because the large particles are mostly limited to stacked membrane areas, while smaller particles are found in similar densities on both Cs and Cu faces (Staehelin, unpublished results; Figs 1 and 2), the large particles are the logical choice for Photosystem II complexes and the smaller ones for Photosystem I complexes. A similar proposal was put forward by Arntzen et al. [29] but has not been further tested since then. However, regardless of which type of particle might correspond to which photosystem, the observations reported in this paper support both the notion that thylakoid membrane stacking might increase the interactions between the two photosystems and suggest a mechanism for increased interactions.

Simple geometrical considerations indicate that unstacked membranes only permit lateral interactions, i.e. within the plane of each membrane, between presumed elements of the two photosystems. By bringing adjacent membranes into direct contact, additional vertical interactions between components in opposing membranes become possible. Our present finding that under such conditions particles of different sizes link up with each other is consistent with the idea that stacking maximizes interactions between the two photosystems by enabling Photosystem II and Photosystem I complexes of adjacent membranes to make functional contact, thus supplementing their lateral interactions with vertical ones. To this end, our results suggest that stacking should both increase spillover between the photosystems and enhance the quantum yield at low light intensities. Since the bonding between stacked thylakoid membranes is increased by higher concentrations of ions, Murakami and Packer [5] have suggested that membrane stacking involves hydrophobic interactions. Should this be the case, then elements of the two photosystems could conceivably be linked through a medium with similar properties as found within the plane of each membrane.

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REFERENCES

1 Anderson, J. M. and Boardman, N. K. (1966) Biochim. Biophys. Acta 112, 403–421
2 Murata, N. (1969) Biochim. Biophys. Acta 189, 171–181
3 Murata, N. (1971) Biochim. Biophys. Acta 245, 365–372
4 Murakami, S. and Packer, L. (1970) J. Cell Biol. 47, 332–340
5 Murakami, S. and Packer, L. (1971) Arch. Biochem. Biophys. 146, 337–347
6 Phung Nhu Hung, S., Lacourly, A. and Sarda, C. (1970) Z. Pflanzenphysiol. 62, 1–16
7 Gross, E. L. (1971) Arch. Biochem. Biophys. 147, 77–84
8 Gross, E. L. (1972) Arch. Biochem. Biophys. 150, 324–329
9 Sun, A. and Sauer, K. (1972) Biochim. Biophys. Acta 256, 409–427
10 Wang, A. Y. I. and Packer, L. (1973) Biochim. Biophys. Acta 305, 488–492
11 Berg, S., Dodge, S., Krogman, D. W. and Dilley, R. A. (1974) Plant Physiol. 53, 619–627
12 Gross, E. L. and Hess, S. C. (1974) Biochim. Biophys. Acta 339, 334–346
13 Vandermeulen, D. L. and Govindjee (1974) Biochim. Biophys. Acta 368, 61–70
14 Sane, P. V., Goodchild, D. J. and Park, R. B. (1970) Biochim. Biophys. Acta 216, 162–178
15 Arntzen, C. J., Dilley, R. A., Peters, G. A. and Shaw, E. R. (1972) Biochim. Biophys. Acta 256, 85–107
16 Goodenough, U. W. and Staehelin, L. A. (1971) J. Cell Biol. 48, 594–619
17 Pendland, J. C. and Aldrich, H. C. (1973) J. Cell Biol. 57, 306–314
18 Staehelin, L. A. (1974) Int. Rev. Cytol. 39, 191–283
19 Park, R. B. and Biggins, J. (1964) Science 144, 1009–1011
20 Park, R. B. and Pfeifhofer, A. O. (1969) J. Cell Sci. 5, 299–311
21 Sleytr, U. B. (1974) Proceedings of the Eighth International Congress on Electron Microscopy, Vol. 2, pp. 30–31
22 Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731
23 Nicolson, G. L. (1974) Int. Rev. Cytol. 39, 89–190
24 Singer, S. J. (1974) Annu. Rev. Biochem. 43, 805–833
25 Vanderkooi, G. (1974) Biochim. Biophys. Acta 344, 307–345
26 Branton, D. and Park, R. B. (1968) J. Ultrastruct. Res. 19, 283–303
27 Miller, K. R. and Staehelin, L. A. (1973) Protoplasma 77, 55–78
28 Ojakian, G. K. and Satir, P. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2052–2056
29 Arntzen, C. J., Dilley, R. A. and Crane, F. L. (1969) J. Cell Biol. 43, 16–31
30 Goodchild, D. J. and Park, R. B. (1971) Biochim. Biophys. Acta 226, 393–399
31 Akoyunoglou, G. and Argyroudi-Akoyunoglou, J. (1974) FEBS Lett. 42, 135–140