REVERSIBLE PARTICLE MOVEMENTS ASSOCIATED WITH UNSTACKING AND RESTACKING OF CHLOROPLAST MEMBRANES IN VITRO

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ABSTRACT

Freeze-fracture and freeze-etch techniques have been employed to study the supramolecular structure of isolated spinach chloroplast membranes and to monitor structural changes associated with in vitro unstacking and restacking of these membranes. High-resolution particle size histograms prepared from the four fracture faces of normal chloroplast membranes reveal the presence of four distinct categories of intramembranous particles that are nonrandomly distributed between grana and stroma membranes. The large luminal surface particles show a one to one relationship with the EF-face particles. Since the distribution of these particles between grana and stroma membranes coincides with the distribution of photosystem II (PS II) activity, it is argued that they could be structural equivalents of PS II complexes. An interpretative model depicting the structural relationship between all categories of particles is presented.

Experimental unstacking of chloroplast membranes in a low-salt medium for at least 45 min leads to a reorganization of the lamellae and to a concomitant intermixing of the different categories of membrane particles by means of translational movements in the plane of the membrane. In vitro restacking of such experimentally unstacked chloroplast membranes can be achieved by adding 2–20 mM $MgCl_2$ or 100–200 mM NaCl to the membrane suspension. Membranes allowed to restack for at least 1 h at room temperature demonstrate a resegregation of the EF-face particles into the newly formed stacked membrane regions to yield a pattern and a size distribution nearly indistinguishable from the normally stacked controls. Restacking occurs in two steps: a rapid adhesion of adjoining stromal membrane surfaces with little particle movement, and a slower diffusion of additional large intramembranous particles into the stacked regions where they become trapped. Chlorophyll a:chlorophyll b ratios of membrane fractions obtained from normal, unstacked, and restacked membranes show that the particle movements are paralleled by movements of pigment molecules. The directed and reversible movements of membrane particles in isolated chloroplasts are compared with those reported for particles of plasma membranes.

In higher plants and green algae the photosynthetically active membranes of chloroplasts, the thylakoids, possess the capacity to fuse over long distances. Such regions are referred to as "grana" in higher plants and as "stacks" in green algae, while the nonfused or unstacked areas are known as "stroma" membranes.

During the last decade, numerous studies have demonstrated that thylakoids can be fractionated into grana and stroma membrane regions. This can be achieved by differential centrifugation of isolated chloroplasts broken with digitonin (2, 7, 17), with sonication (21), or with a French pressure cell (8, 37). Testing of the fractions has revealed that the lighter stroma membranes contain essentially only photosystem I (PS I) activity and a corresponding high chlorophyll (chl) a:chl b ratio; grana fractions have both PS I and PS II and a low chl a:chl b ratio. Thus, it is generally assumed that PS II complexes are essentially limited to grana membrane regions, whereas PS I complexes are found in both grana and stroma membranes.

Complementing these studies are others showing a dependence of the structure and function of chloroplasts on their ionic environment. To this end, appropriate concentrations of mono- and divalent cations can stimulate the rate of the Hill reaction, the efficiency of photophorylation, and the light-induced uptake of protons, consistent with the idea that cations control the coupling of redox reactions to the energy-conserving mechanism of the membrane (19, 38). In addition, Izawa and Good (20) have found the organization of thylakoid membranes to be controlled by cations as evidenced by the conversion of isolated chloroplasts into a grana-free membrane system under low-salt conditions. This effect is partially reversed by adding salts. Hence, cations seem to be required to stabilize the stacking of thylakoids in grana.

As demonstrated by Goodenough and Staehelin (18) in their freeze-fracture study of chloroplast membranes of *Chylamydomonas,* the functional differentiation of thylakoids is matched by a nonrandom distribution of intramembranous particles between stacked and unstacked membrane regions. In particular, they noted that in freeze-fracture replicas stacked regions could be recognized by their complement of regularly spaced, large intramembranous particles 160 ± 10 Å in diameter that remain associated with the luminal membrane leaflet during freeze-fracturing. Earlier, the equivalent particles of spinach chloroplasts were referred to as quantasome cores (34) or PS II markers (7).

Upon suspension of *Chlamydomonas* chloroplasts in a low-salt medium and disintegration of the stacks into single thylakoids, the particles of the stacked regions disperse laterally and become intermixed with those of unstacked stroma regions (32). When Ojakian (31) added divalent ions to such experimentally unstacked membranes of wild-type chloroplasts, he observed the reformation of membrane stacks but no reaggregation of the large intramembranous particles into these regions.

The present investigation was initiated to develop in vitro conditions capable of bringing about complete restacking of experimentally unstacked thylakoids, i.e. conditions that would lead not only to the formation of contact regions between adjacent membranes but also to the redistribution of the randomized intramembranous particles into the restacked membrane regions. While pursuing these goals, using spinach chloroplast membranes as a model system, we soon became aware of the lack of a complete qualitative description of the freeze-fracture and freeze-etch morphology of normal higher-plant chloroplasts in the literature. We therefore systematically re-analyzed both the fracture face and the true surface morphology of isolated spinach chloroplast membranes in various isolation media and found striking new correlations between internal and external membrane features. These results, in turn, have enabled us to obtain unambiguous, direct proof for reversible movements of membrane components upon unstacking and restacking of the thylakoid membranes, providing a new means for routinely assaying such movements.

MATERIALS AND METHODS

Isolation of Chloroplasts

Fresh spinach leaves were obtained from a local market, deveined, and macerated in cold isolation buffer (either 0.35 M NaCl, 0.05 M Na phosphate buffer, pH 7.3, 0.001 M EDTA; or 0.3 M sucrose, 0.05 M Na phosphate buffer pH 7.3, 0.01 M KCl) in a Waring blender for 15–20 s. The cell homogenate was passed through several layers of gauze, then centrifuged for 2 min at 300 *g*. The pellet from this spin was discarded. The supernate was then centrifuged at 2,000 *g* for 10 min, the supernate discarded, and the pellet resuspended in either 0.3 M sucrose, 0.05 M Na phosphate buffer,

pH 7.3, or 0.15 M KCl, 0.05 M Na phosphate buffer, pH 7.3. This suspension was centrifuged at 1,000 *g* for 10 min. The resulting pellet contained nearly pure chloroplasts as shown by thin-section electron microscopy. All experiments have also been duplicated and confirmed with pea chloroplasts isolated from leaves of dwarf peas grown under fluorescent lamps on vermiculite soaked with one-half strength Hoagland's solution.

Unstacking and Restacking of Thylakoids

Techniques used for unstacking and restacking isolated chloroplasts are modifications of methods first described by Izawa and Good (20). For the unstacking of isolated thylakoids, the membranes were washed once in 50 mM and then twice in 5 mM Na-Tricine, pH 7.6, over the course of 1 h in the cold. Membranes unstacked by the procedure and being used for restacking experiments were suspended in either a 5-mM or a 50-mM Tricine buffer, pH 7.6, containing the desired concentration of Mg^{++} (0.1–20 mM $MgCl_2$) or Na^{+} (10–200 mM NaCl) ions. The restacking process was allowed to proceed either in the cold or at room temperature for periods of 5 min to 2 h. Where timing of the restacking process was critical, the reaction was stopped by the addition of 0.5% glutaraldehyde to the restacking medium. In control experiments, it was shown that experimentally unstacked thylakoid membranes could be prevented from restacking in the presence of Mg^{++} ions by prior fixation with 0.5% glutaraldehyde. Since glutaraldehyde fixation also leads to a general reduction in particles that cleave with EFs and EFu faces (Staehelin, unpublished observations), no quantitative particle measurements were made on samples subjected to this treatment. However, the general findings of the timing experiments have been confirmed in unfixed samples stabilized by freezing at time intervals approximating those used in the other experiments. All experiments have been repeated at least three times.

Chlorophyll Measurements On Subchloroplast Fractions

Fractions of stacked (grana) and unstacked (stroma) thylakoids were prepared by a modified procedure of Arntzen et al. (8) by passing isolated membranes four times through a French press at 4,000 lb/in² pressure, followed by centrifugations at 2,000 *g* · 10 min (pellet discarded), at 40,000 *g* · 30 min for grana fractions, and 144,000 *g* · 60 min for stroma fractions (designated 40 K and 144 K fractions). The 40 K and 144 K pellets were resuspended in water and their respective chl a and chl b concentrations were determined by the spectrophotometric method of Arnon (5). The chl a:chl b ratios shown in Table II represent average values of five different experiments.

Freeze-Fracturing and Freeze-Etching

Membrane samples for freeze-fracturing were pelleted and frozen either in their reaction medium or in the reaction medium plus 30% glycerol. Samples used for etching experiments were pelleted and frozen in the reaction mixture if the total concentration of solutes was less than 10 mM; otherwise, the membranes were fixed with 0.5% glutaraldehyde for 10 min and then washed twice in 5 mM Tricine, pH 7.6, with or without 2 mM $MgCl_2$ before being frozen in the washing solution.

Replicas were prepared according to standard procedures on a Balzers BA360 freeze-etching apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). Specimens were freeze-fractured at −106°C; etching was carried out at −100°C for 30 s to 5 min.

Measurements and Calculations of Structural Membrane Parameters

Particle sizes were determined from micrographs taken at × 40,000 and enlarged to × 200,000 and viewed through a × 7 objective lens equipped with a micrometer grating. The width of the shadow of a given particle was measured over the shadowed half of the particle. Where the edge of the shadow appeared fuzzy or irregular, a minimum width was always taken. Between 350 and 800 particles were measured in large continuous areas on 3–6 micrographs for each histogram of particle size distribution (Figs. 3 and 18). Distribution counts were made on 4–10 micrographs of large, flat membrane regions similar to Figs. 2, 6, and 11, and enlarged to × 100,000 by imposing a transparent sheet of graph paper and counting all particles within quadrants on the paper. Between 3 and 6 μm^2 of each type of fracture face or membrane surface was analyzed.

The percentage of stacked membrane regions was determined by measuring with a map reader six randomly chosen micrographs of thin sections. Formulas of Ojakian and Satir (32) were used to calculate the expected particle size distributions in Fig. 18 and the particle densities in Table I.

RESULTS

Freeze-Etch Nomenclature of Chloroplast Membranes

In the present communication, we have applied for the first time the new nomenclature of freeze-etched membranes (10) to chloroplast membranes. This new nomenclature is based on the fact that all biological membranes consist of two leaflets, a protoplasmic (P) and an exoplasmic (E) leaflet, and that each leaflet possesses a fracture face (F) and a true surface (S), thus leading to the designations PF and EF for fracture faces and PS and ES for surfaces. Diagram Fig. 1 has been included in this report to illustrate how the nomenclature applies to the different types of fracture faces and etched surfaces of stacked and unstacked regions of thylakoid membranes, and to

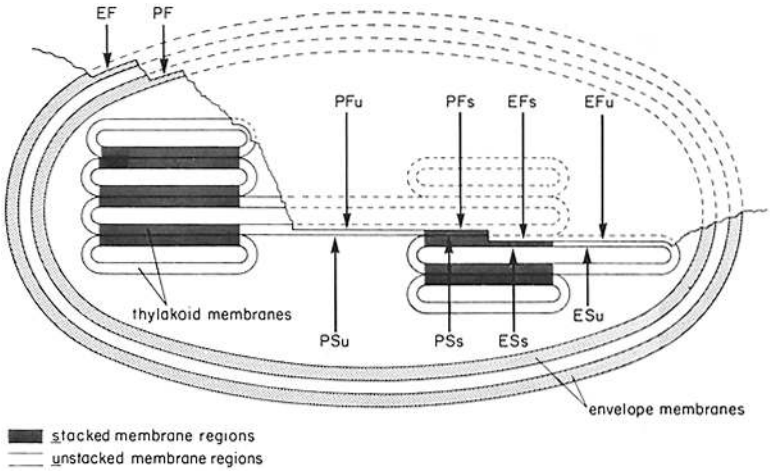


FIGURE 1 Diagram illustrating the freeze-fracture and freeze-etch nomenclature of chloroplast membranes used in this paper, which is based on the general freeze-etch nomenclature proposed by Branton et al. (10). *P* refers to the protoplasmic (cytoplasmic, stroma) leaflet and *E* to the exoplasmic (external, luminal) leaflet of biological membranes. *F* stands for internal fracture faces of membranes, *S* for the surfaces of membranes that can be observed after freeze-etching or deep-etching of membranes frozen in a suitable, "etchable" medium. *u* and *s*, finally, designate unstacked or stacked membrane regions as proposed by Goodenough and Staehelin (18).

help the reader relate the many different freeze-etch images presented here to the images seen in conventional thin sections. The fracture face formerly known as Bs face (18) is now EFs. The other designations of Goodenough and Staehelin (18) have changed as follows: Bu → EFu, Cu → PFu, Cs → PFs, Au → PSu, As → PSs, Du → ESu, Ds → ESs.

Normal Thylakoid Structure

A typical freeze-fracture image of isolated spinach thylakoids is illustrated in Fig. 2. Four distinct types of membrane fracture faces can be recognized, two derived from stacked (grana) regions (EFs and PFs), and two from unstacked (stroma) regions (EFu and PFu). Since their morphology corresponds closely to the morphology of wild-type *Chlamydomonas* chloroplast membranes described in detail by Goodenough and Staehelin (18), they will be discussed only briefly in the following paragraphs.

The EFs face is the most distinctive thylakoid fracture face due to its dense population of large, clearly spaced particles on a relatively smooth background matrix. It is evident from the particle size histogram Fig. 3*a* that, although the particles range in size from 80 Å to 200 Å, the majority fall into two distinct size categories: <140 Å and >140 Å.

The PFs face—like the complementary EFs face—is characteristic of regions where the membranes are in a stacked configuration. The PFs face appears to be quite uneven, largely because it contains a mixture of large pits and small particles. As illustrated by the histogram Fig. 3*b*, the PFs particles are both fairly uniform in size and on the average smaller (average diameter ~80 Å) than any of the other thylakoid fracture face particles.

The EFu face is derived from unstacked membrane regions and is often seen to be continuous with an EFs face. It carries only a sparse population of particles, the average size of which is similar to the smaller size category of EFs face particles (compare histograms Fig. 3*a* and 3*c*). The background matrix is marked by numerous pits.

The PFu face is derived from fracturing the membrane in unstacked regions, and is complementary to the EFu face. The particles on this face appear more densely packed than those of any other face. The particle-size histogram Fig. 3*d* indicates that they fall into two size categories, <100 Å and >100 Å in diameter. It is interesting to note that the average particle diameter in the smaller size category (81.8 Å) is nearly identical to the average size of the PFs particles (82.2 Å; compare Fig. 3*b* and 3*d*).

While most of the grana stacks of spinach chloroplasts are interconnected by tubular strands of

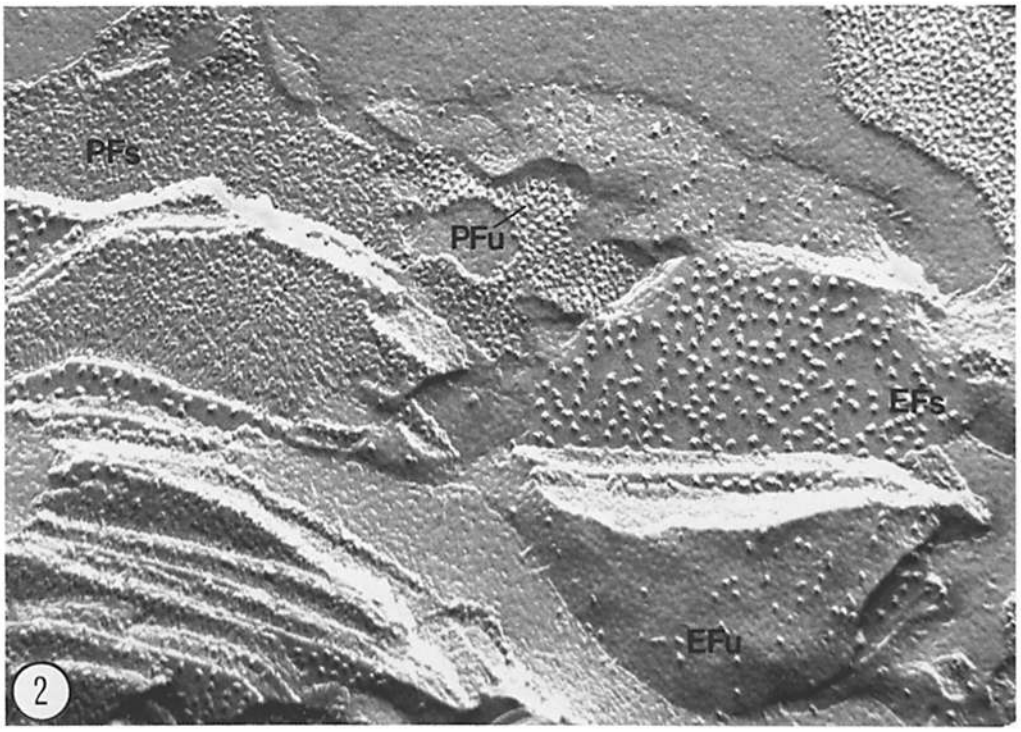


FIGURE 2 Freeze-cleaved isolated thylakoids of spinach. The flat, partly circular membranes of two grana stacks (left and right) appear interconnected by more tubular membranes of a stroma lamella. The complementary type fracture faces marked *PFs* and *EFs* are characteristic of stacked membrane regions, while the faces *PFu* and *EFu* belong to unstacked membrane regions (see also diagram Fig. 1 for chloroplast membrane nomenclature). Note the distinct aggregate of large 160-Å particles on the *EFs* face. × 87,000.

stroma lamellae, it is sometimes possible to find in isolated chloroplasts an occasional sheetlike thylakoid depicting alternating stacked and unstacked membrane regions (Figs. 4–6). In the present study such thylakoids have proved particularly helpful for elucidating the relationship between particles seen on fracture faces (Fig. 4) and those visible on true membrane surfaces (Figs. 5–7).

As seen in Fig. 4, most stacked membrane regions (PFs and EFs areas) have roughly circular profiles. A similar distribution of membrane patches marked by aggregates of large particles (ESs areas) can be recognized in luminal surface views of thylakoid membranes revealed by deep-etching (Fig. 5). That these patches of large particles correspond to stacked membrane regions is supported not only by the size, form, and distribution of the patches but also by the presence of funnel-shaped depressions around their margins (arrows, Fig. 5; compare also with Figs. 4 and 23), which represent openings of the thin tubular connections between the continuous lumina of the stroma and grana thylakoids. The surrounding, unstacked membrane regions show little surface texture when viewed in low-power micrographs (Fig. 5).

At higher magnifications, further structural details of the luminal surface of thylakoid membranes may be discerned. Close examination of the large particles in the ESs regions in Figs. 6, 8, and 9 reveals the presence of small subunits. Where the particles are randomly distributed (Figs. 6, 8) the majority display four subunits, although some smaller ones appear to possess as few as two and some larger ones as many as six. However, when the particles become organized into a geometrical lattice (usually a rectangular array, Figs. 6, 9), all particles exhibit four subunits. In such lattices, the individual ESs particles measure approximately 100 Å × 155 Å, the repeating distances along the two axes approximately 175 Å × 204 Å.

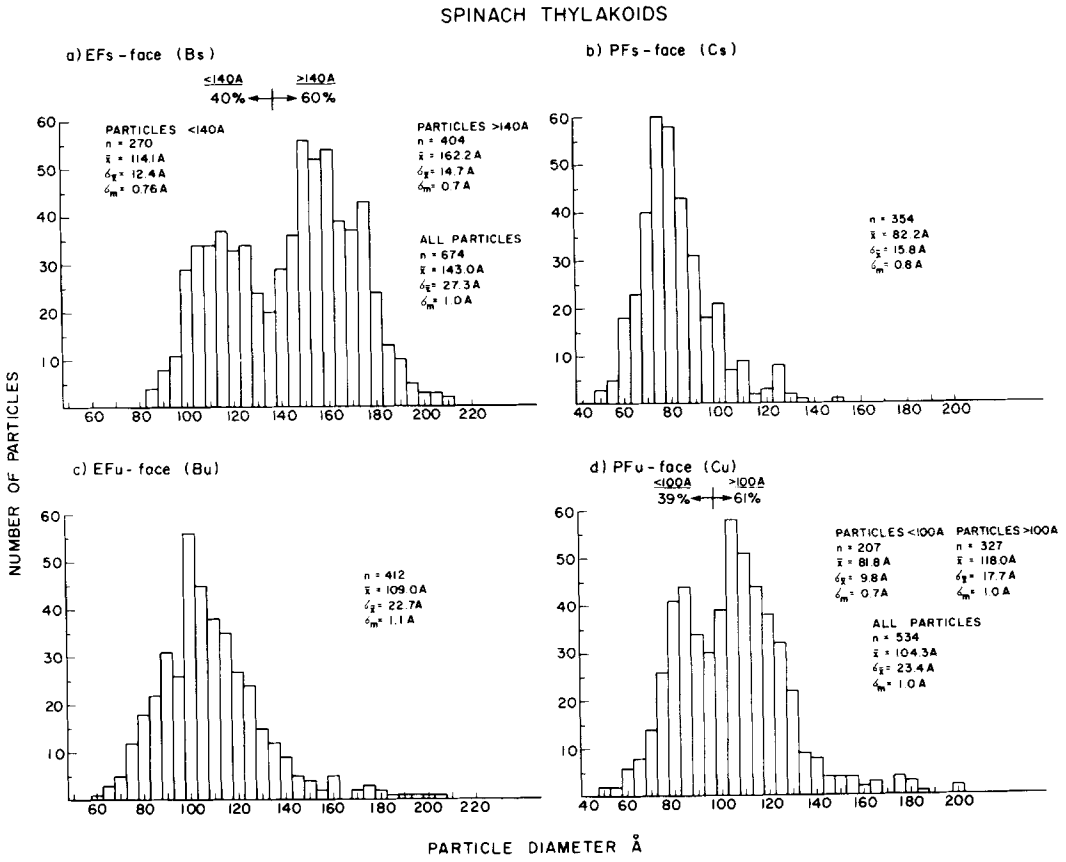
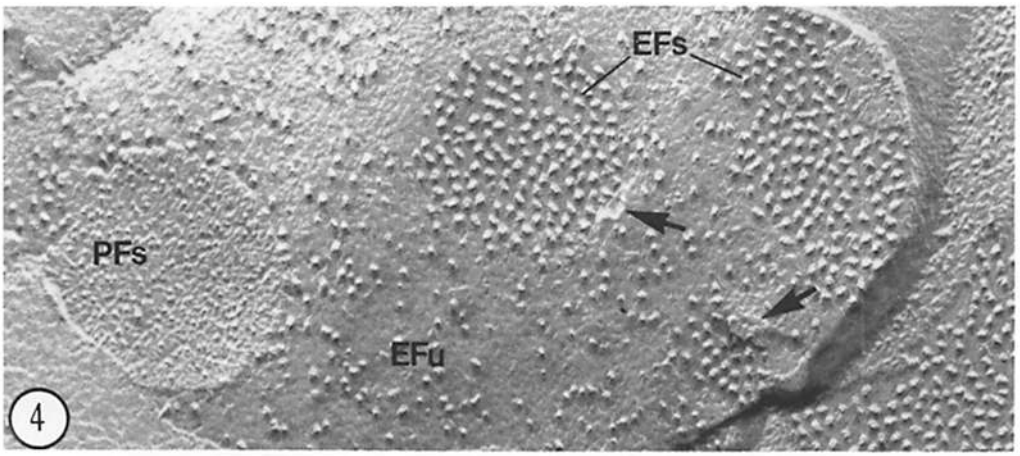


FIGURE 3 Histograms of particle sizes found on the four types of membrane fracture faces of spinach chloroplasts. The histograms are labeled *a–d*, and each is identified by individual labels.

Several lines of evidence indicate that these ESs particles are identical to the large particles seen in stacked regions of EF faces. Besides being limited to stacked membrane regions and being associated with the luminal membrane leaflet, both types of particles have similar sizes (arrayed EFs particles are uniformly ~160 Å in diameter), are found in similar densities (Table I), and exhibit essentially the same lattice periodicities when arranged in rectangular arrays (175 Å × 204 Å vs. 174 Å × 206 Å). In addition, they display identical changes in their distribution patterns during unstacking and restacking experiments in vitro (see second half of Results). These correlations suggest that the large particles seen in the stacked regions of ES surfaces and of stacked EF faces represent different views of the same membrane component.

Having determined the above correlation between ESs and EFs particles, we addressed ourselves to the question of whether the EFu particles are related to EFs particles, or whether they represent a completely different kind of membrane component. As seen in the particle size histograms (Fig. 3 *a* and *c*), the average EFu particle is only as large as the smaller category of EFs particle. Very few EFu particles have sizes greater than 140 Å, corresponding to the larger EFs particles. Because all fracture face particles seem to be, to some extent, plastically deformed during freeze-cleaving (40), and because we do not know how membrane stacking may affect the cleaving behavior of the particles, it is presently impossible to determine from fracture face images alone whether EFu and EFs particles are related or not. This problem can be solved, however, by examining true membrane surfaces, since the particles seen on such surfaces are not subjected to the same stress conditions. Thus, morphological characteristics of the particles can be used for their identification on etched



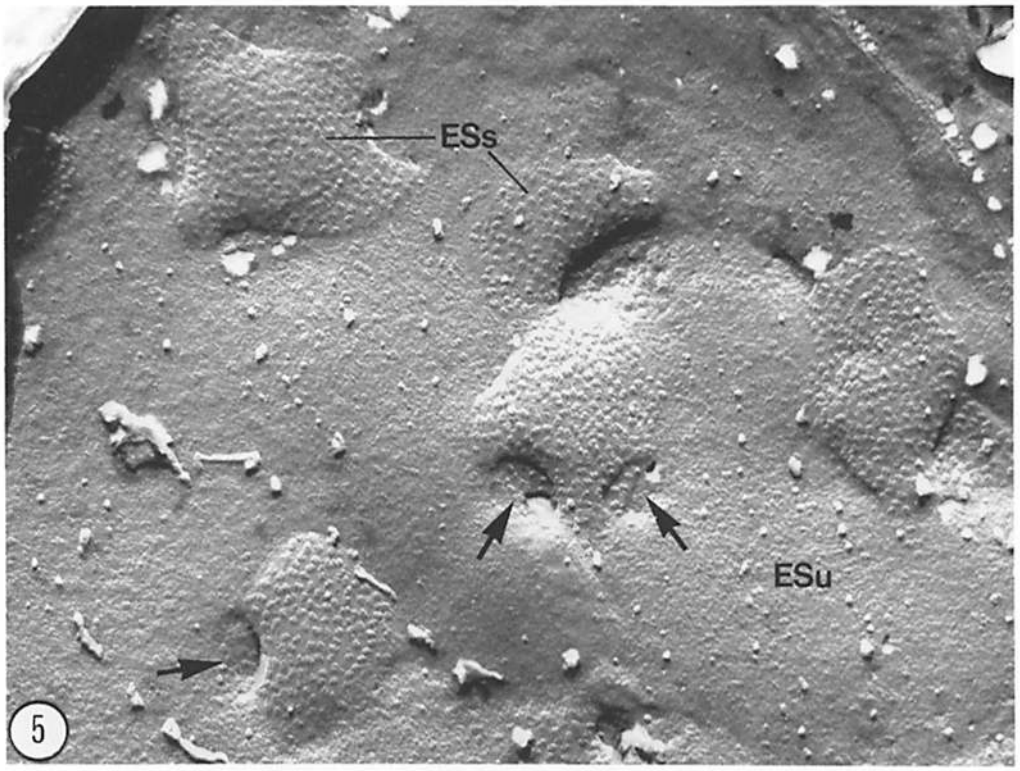


FIGURE 4 Fracture face of a large, sheetlike, and slightly swollen thylakoid depicting both stacked (*EFs*) and unstacked (*EFu*) membrane regions. The round patches of large particles (*EFs* faces) and the single *PFs* face correspond to areas of membrane contact. The arrows point to cross-fractured tubular connections that link the lumina of the stroma and grana thylakoids (compare with Fig. 5). × 93,000.

FIGURE 5 True luminal surface (ES) of a large, sheetlike spinach thylakoid exposed by deep-etching. The patches of large particles (*ESs*)correspond in their size and distribution to the stacked membrane regions associated with the grana. The surrounding, unstacked membrane regions (*ESu*) appear, at this magnification, relatively smooth. Funnel-shaped depressions (arrows) can be recognized around the margins of the stacked membrane areas. They correspond to the openings of the thin tubular connections between the lumina of the stroma and grana thylakoids and between adjacent grana thylakoids. × 67,000.

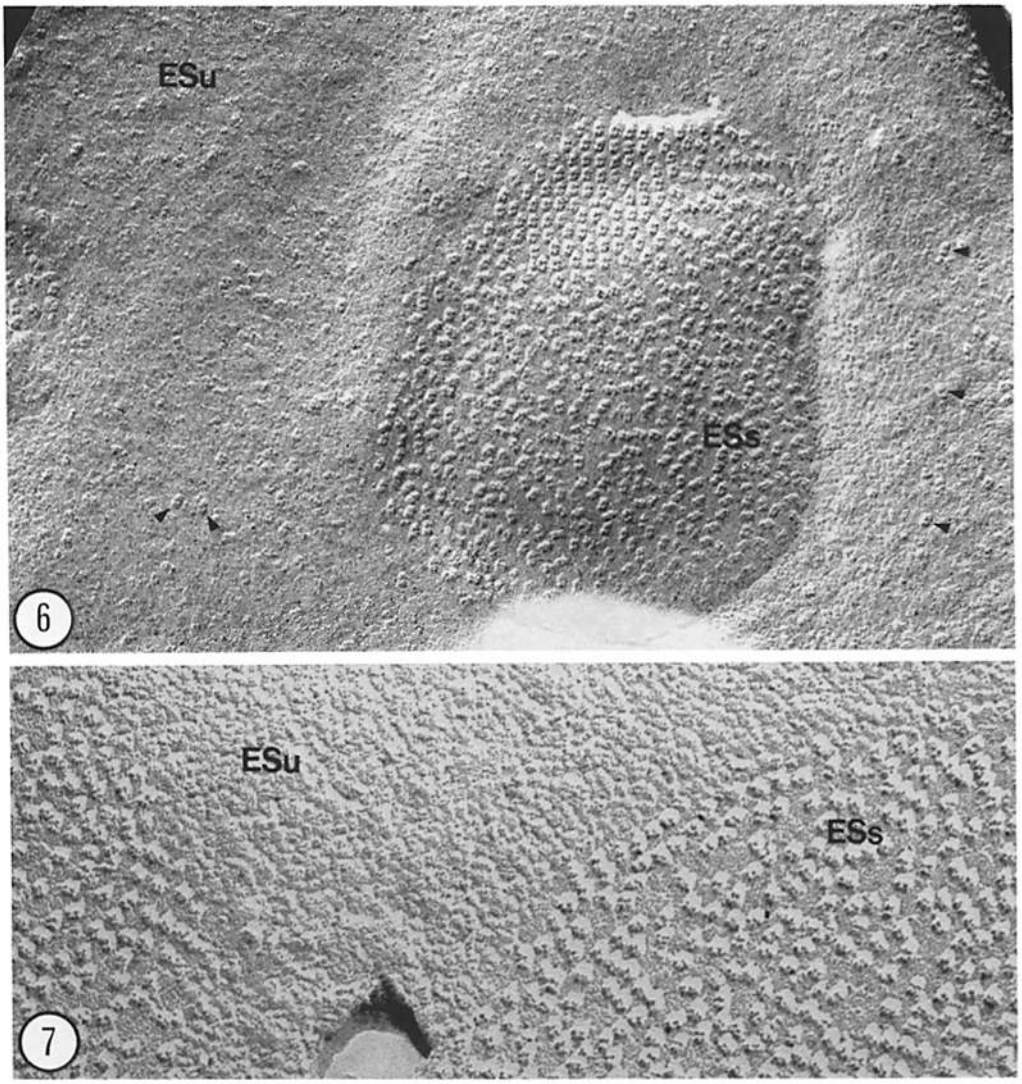


FIGURE 6 Higher magnification of the true inner surface of spinach thylakoid revealed by deep-etching. The stacked membrane region (*ESs*) in the center of the micrograph is clearly delineated from the surrounding unstacked areas (*ESu*) by the closely packed large particles, which protrude from a very smooth background. The particles appear to consist of subunits (mostly four, but sometimes as few as two or as many as six; see also Fig. 8), and can become organized into geometrical patterns (most frequently rows or rectangular arrays). The surface of the unstacked membrane regions is nearly, but not completely devoid of the large particles that tend to aggregate in the stacked membrane regions. The arrowheads point to a few of these particles that are somewhat difficult to recognize on the pebbly surface of the background material. × 116,000.

FIGURE 7 Micrograph of the true inner surface of a thylakoid chosen to illustrate the difference in surface texture of the membrane matrix in stacked (*ESs*) and unstacked (*ESu*) membrane areas. Notice the pebbly surface of the ESu regions and the smooth background material in the ESs areas (see also Fig. 8). × 127,000.

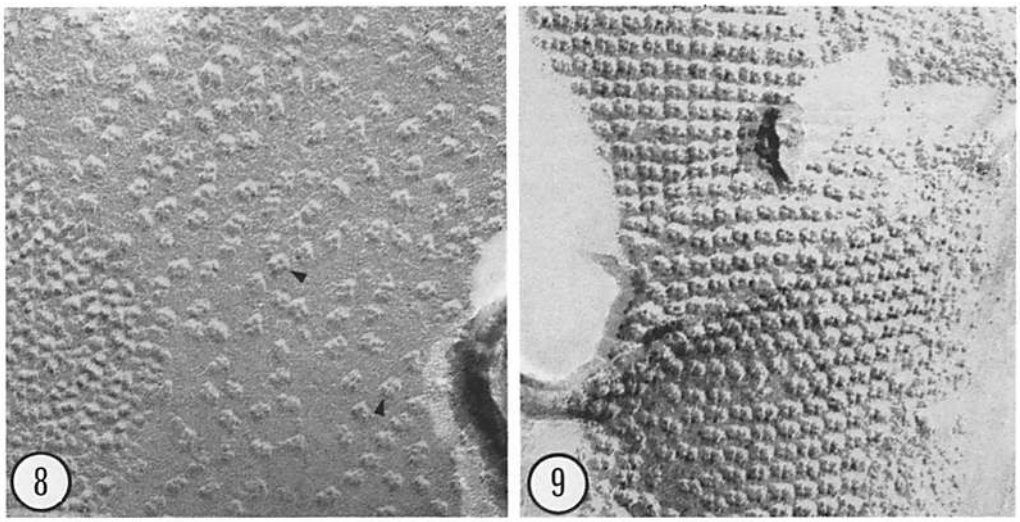


FIGURE 8 Higher magnification of the inner surface of a stacked thylakoid region illustrating the smooth texture of the membrane matrix and the diversity in size, shape, and orientation of the large, randomly distributed particles. The arrowheads point to particles that clearly exhibit four subunits. × 165,000.

FIGURE 9 Similar membrane surface area (ES) as shown in Fig. 8 but with large particles organized into a quasi-square lattice. Notice the regular size of the particles (compared to Fig. 8) and the alignment of their subunits. × 165,000.

TABLE I

Particle Density (Particles per μm^2 ± SE) on Fracture Faces and Etched Surfaces of Spinach Thylakoid Membranes

Face/surface	Control*	Experimentally unstacked‡	Experimentally restacked§
EFs	1,495 ± 103		1,399 ± 211
ESs	1,588 ± 136		1,426 ± 225
EFu	574 ± 47		610 ± 96
ESu	604 ± 82		560 ± 139
PFs	3,409 ± 265		3,383 ± 343
PFu	3,620 ± 286		3,555 ± 358
EF (measured)		1,129 ± 137	
EF (calculated)		1,167 ± 83	
ES (measured)		1,134 ± 154	
ES (calculated)		1,238 ± 116	

* Washed twice in 2 mM $MgCl_2$, 5 mM Tricine pH 7.6.
‡ Suspended in 5 mM Tricine pH 7.6.
§ 1.5 h in 2 mM $MgCl_2$ at room temperature.

membrane surfaces. With this in mind and knowing the correspondence between EFs and ESs particles, we can examine ESu regions for the presence of ESs-type particles. Careful examination of ESu regions of Fig. 6 shows, indeed, particles (small arrowheads) with the same substructure and the same dimensions as those seen in ESs areas. In addition, the density of these large ESu particles corresponds closely to the density of the EFu particles (Table I). On the basis of these observations, we suggest (*a*) that the EFu and the ESu particles represent two different images of the same membrane component, and (*b*) that all EF face particles contain identical cores as indicated by their identical luminal surface morphology, even though their diameters vary greatly between stacked and unstacked membrane regions. In other words, the membrane components that give rise to the typical large particles in the EFs areas of freeze-fractured thylakoid membranes are probably not unique components of stacked regions: rather, their affinity for stacked over unstacked membrane regions is approximately four times greater. Since roughly 60% of the thylakoids of normal spinach chloroplasts are in a stacked configuration, we can calculate that grana regions contain 80–85% and stroma regions 15–20% of the membrane components giving rise to the EF face particles.

Another important structural difference between stacked and unstacked thylakoid membrane areas, seen in Figs. 6 and 7, is that the surface of the matrix material between the large particles in ESs regions is much smoother than the cobblestone-like surface of ESu regions. A qualitative and quantitative description of the stromal (PSu) surface morphology of spinach thylakoid mem-

branes has already been presented by Miller and Staehelin (28).

Structural Changes Associated with In Vitro Unstacking of Thylakoids

In vitro unstacking of isolated spinach thylakoids can be brought about by washing them in a low-salt medium consisting of 0.05 M Tricine-NaOH, pH 7.6 (20). If the thylakoids are allowed to unstack at 4°C for less than 15 min, the grana membranes become separated, while the overall organization of the grana and stroma lamellae is retained together with the normal distribution of particles between the different areas.[1] In contrast, exposure of isolated chloroplasts to the unstacking medium for 45 min or longer leads not only to separation of the grana membranes but also to reorganization of the lamellae into large, roughly parallel folds (Fig. 10). Concomitantly, both the large EF face and the smaller PF face particles as well as the ES surface particles become completely randomized (Figs. 10, 11). Samples allowed to unstack for intermediate time periods contain a mixture of both types of chloroplasts, e.g., after 30 min of unstacking in the cold, between 40% and 50% of the chloroplasts still possess distinct but separated grana membranes with aggregated large particles.[1] For the restacking experiments reported in the next section, we have used exclusively chloroplasts unstacked for 1 h or longer and possessing large, foldlike thylakoids with randomly distributed particles.

The intermixing of the intramembranous particles of stacked and unstacked membrane regions during experimental unstacking of thylakoid membranes was first characterized by Ojakian and Satir (32) for *Chlamydomonas* chloroplasts. These authors observed a general conservation of particle sizes during this process, although some small shifts in the size of some EF face particles were noted when they compared the measured sizes with the value calculated from the intermixing of EFu and EFs particles in the appropriate proportions. The shifts, however, proved statistically insignificant ($P = 0.8$). In light of these findings, we have carefully analyzed the EF face particle size histogram of experimentally unstacked spinach thylakoids (Fig. 18*a*), to determine whether the same conservation of particle sizes occurred in our system. In contrast to the *Chlamydomonas* results of Ojakian and Satir (32), our refined spinach EF-face histogram reveals a small but statistically significant increase in size of the <140 Å particles ($P = 0.05$) and a similarly small but highly significant decrease in the diameter of the particles >140 Å ($P < 0.001$) upon unstacking. However, because the shifts are in opposite directions and cancel each other, the average size of all EF face particles remains constant.

While unstacking for 1 h leads to the dispersal of the large ES surface particles, a second membrane component frequently becomes organized into small square arrays (Figs. 11, 12). The formation of these square arrays (repeating distance ~130 Å) has been observed in eight of our unstacking experiments over a 6-mo period, but to date we have not been able to find conditions that will cause their appearance on a regular basis. Glycerination or restacking of unstacked membranes, however, leads to their disappearance. On ES surfaces the particles making up the square arrays appear square, flat-topped, and very closely packed. Unstacked thylakoid preparations that reveal square arrays of particles on their ES surfaces also reveal similarly distributed small square arrays of particles or pits on their fracture faces (Figs. 13, 14), thus indicating that the crystallization process involves both surface and internal membrane components. Although the significance of these arrays is presently unknown, we have included this brief description of some of their features because they have appeared in a sizable number of our preparations and have also been observed in the laboratory of R. Park in Berkeley (personal communication).

Since changes in the stromal (PS) surface morphology of thylakoids caused by in vitro unstacking of the membranes have already been described by Miller and Staehelin (28), they are not reported here.

Structural Changes Associated with Restacking of Experimentally Unstacked Thylakoids

Besides demonstrating that chloroplasts can be unstacked in a low-salt medium containing Tricine buffer, Izawa and Good (20) also reported that experimentally unstacked thylakoids can be restacked in the presence of suitable concentrations of divalent or monovalent ions. Figs. 15 and 16

[1] Staehelin, L. A. Manuscript in preparation.

FIGURE 10 Spinach thylakoids freeze-fractured after unstacking in a low-salt Tricine buffer and infiltration with a 30% glycerol solution. All particles on the fracture faces appear randomly distributed, and no distinction between formerly stacked and unstacked membrane regions can be made. The particle density on the EF face is intermediate between EFs and EFu faces (Figs. 2 and 4). Compare also with Fig. 11. × 61,000.

illustrate two aspects of spinach thylakoids unstacked in vitro, and then restacked by suspending the membranes in a 2 mM $MgCl_2$, 5 mM Tricine, pH 7.6, solution for 1 h at room temperature. The cross-fractured, restacked membrane regions seen in Fig. 15 resemble closely those of normally stacked thylakoids, except that individual stacked regions are more variable in size and form, and that the individual thylakoids in a grana stack are less precisely aligned. Thin sections through such preparations show approximately 90–95% of the chloroplasts to possess extensive and near normal-looking stacked membrane regions. For unknown reasons, the remaining chloroplasts appear only slightly stacked or not stacked at all. When these latter chloroplasts are omitted from the measurements of the percentage of stacked membrane regions in restacked chloroplasts, one obtains a value of approximately 60%, which compares favorably with the 64.5% of control plastids. In face views (Fig. 16), the restacked membranes exhibit the same type of segregation of fracture face particles as found in control chloroplasts. This is most evident on the EF faces, which demonstrate very clearly that restacking of experimentally unstacked thylakoids under appropriate conditions can lead to a similar extent of aggregation of large particles into EFs regions as in controls (compare Figs. 2 and 4 with Fig. 16, and see Table I). Analysis of the particle size histograms (Fig. 18*b*, *c*) reveals that, in the experimentally restacked membranes, the segregation of particle size categories into EFs and EFu regions closely parallels their distribution in control membranes. The statistically significant differences between the experimentally restacked and the control thylakoids seem to reflect only a slight enhancement of the normal segregation patterns, i.e. in experimentally restacked membranes the average EFu particle appears slightly smaller and the average EFs particle slightly larger than in control specimens (Fig. 18*b*, *c*).

Concomitant with the stacking-induced resegregation of the EF face particles, a redistribution of the large, subunit-containing particles of the ES surfaces into characteristic ESs regions can also be observed (Fig. 17). This again supports our proposal that the two types of particles represent

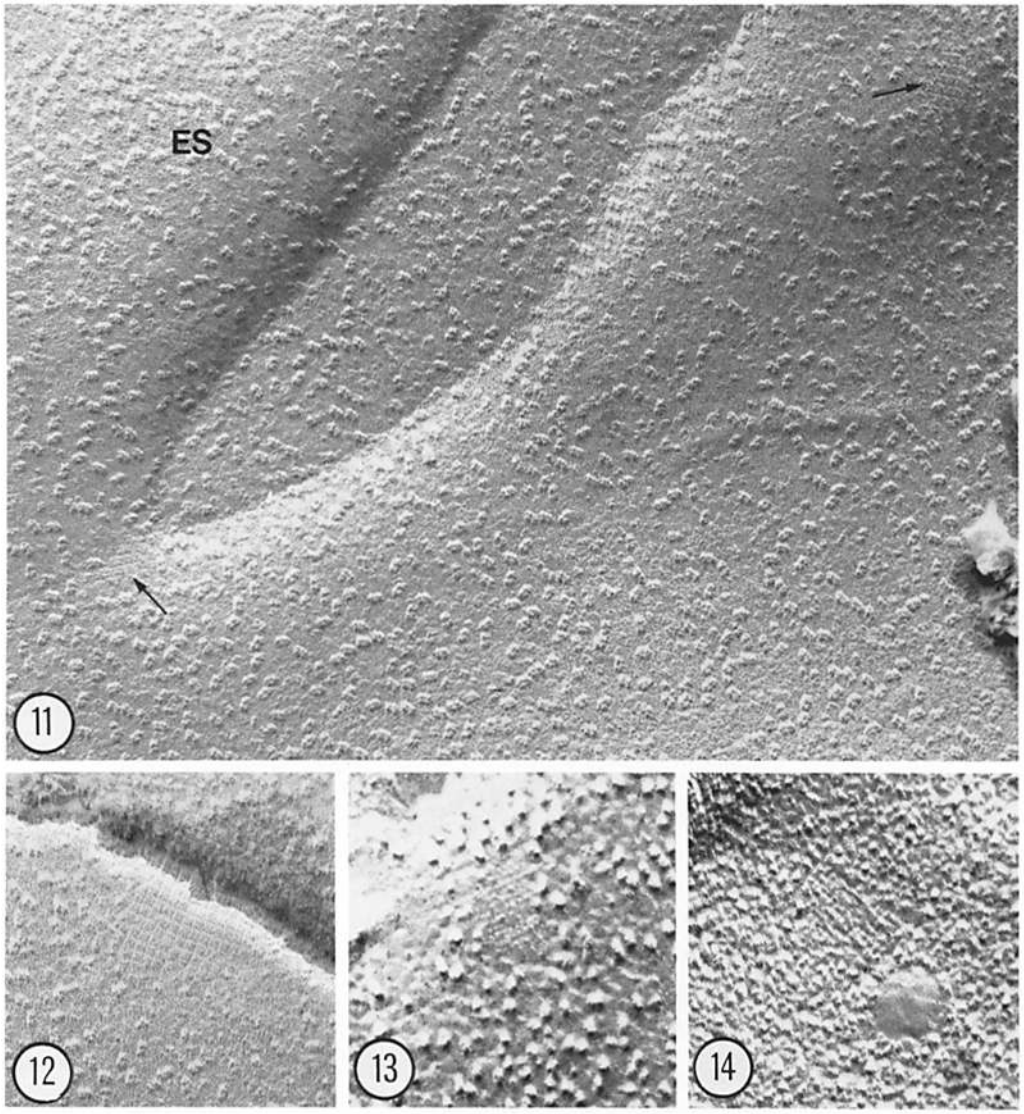


FIGURE 11 True inner ES-surface of a spinach thylakoid after unstacking in a low-salt Tricine buffer in vitro. The large particles that are aggregated into patches under normal conditions (Figs. 5 and 6) appear randomly distributed over the whole membrane surface. Thus the dispersal of ES-surface particles due to unstacking matches the dispersal of EF-face particles in Fig. 10. In between the large particles, several small patches of flat-topped particles in square arrays (arrows) can be recognized (see also Fig. 12). × 108,000.

FIGURE 12–14 Micrographs showing different aspects of the square arrays of flat-topped particles that appear after unstacking of thylakoid membranes in vitro (Fig. 11). All micrographs depict membranes frozen in dilute unstacking medium (5 mM Tricine, pH 7.6) without the addition of glycerol.

FIGURE 12 Inner ES-surface of an unstacked thylakoid depicting a rather large square array of particles (periodicity, ~130 Å). Around the margin of the array several apparently incomplete square particles can be detected, suggesting that the particles contain subunits. × 112,000.

FIGURE 13 EF fracture face of an unstacked thylakoid showing a small patch of depressions organized into a square array. Since such patches only appear in specimens of unstacked membranes that exhibit upon deep-etching square arrays of the type shown in Fig. 12, it is suggested that the two types of square arrays seen in Fig. 12 and this micrograph represent different views of the same structure. × 112,000.

FIGURE 14 PF fracture face of an unstacked thylakoid showing a patch of small particles organized into a difficult to recognize square array. This square array of particles probably represents the complementary-type structure to the array of pits shown in Fig. 11. × 112,000.

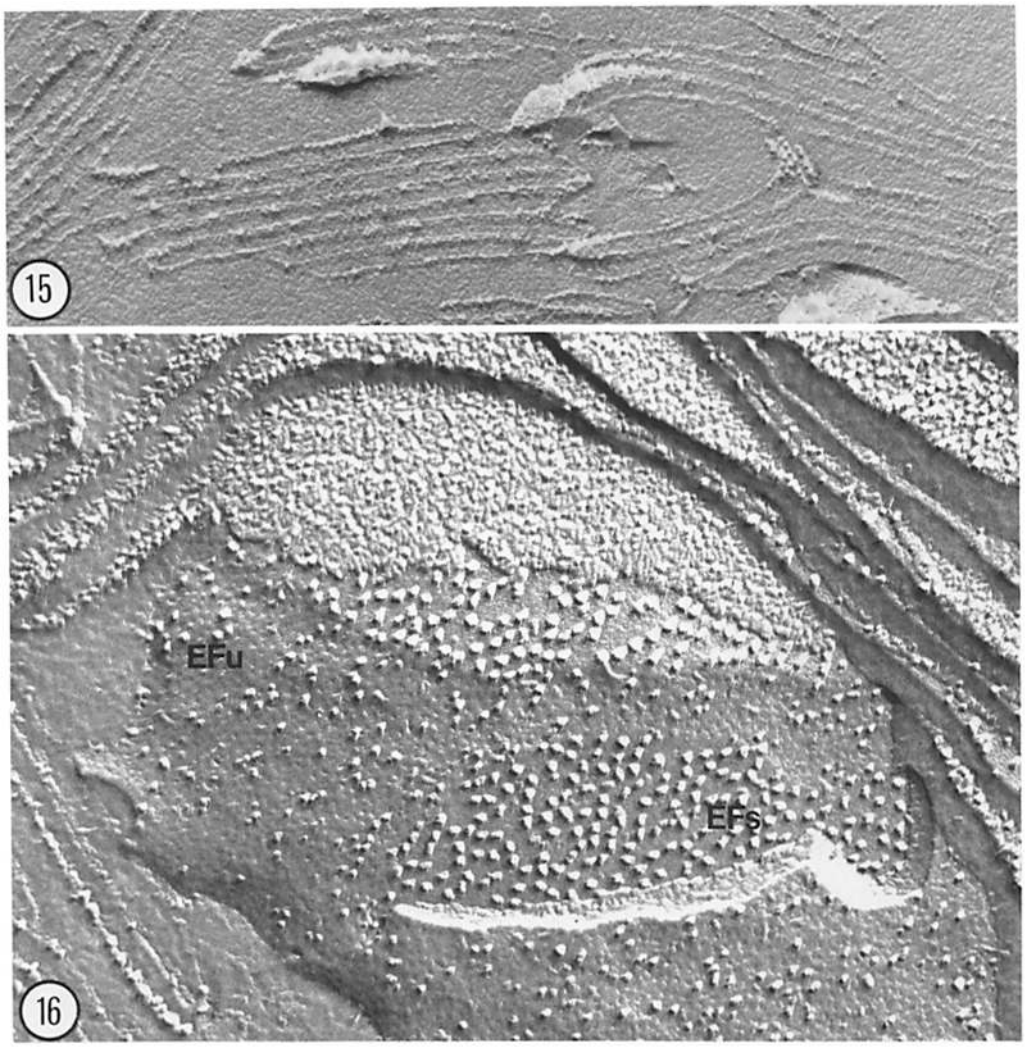


FIGURE 15 Cross-fractured thylakoid membranes unstacked and then restacked in vitro by the addition of divalent cations (2 mM $MgCl_2$) in 5 mM Tricine buffer pH 7.6, 1 h at room temperature; see Materials and Methods for further details. The stacked membrane regions appear essentially similar to those of normally stacked thylakoids, except that the average stacked region is more extensive in area and that the grana are less precisely aligned. × 60,000.

FIGURE 16 Fracture faces of spinach thylakoid membranes unstacked and then allowed to restack in vitro for 1 h in a 2 mM $MgCl_2$ solution as described for Fig. 15. Most of the EF-face particles have resegregated into the stacked (*EFs*) membrane regions; the *EFu* areas are largely depleted of such particles. These EFs and EFu faces appear identical to those of the controls (Fig. 2). × 95,000.

different aspects of the same membrane component.

After the demonstration that restacking of experimentally unstacked chloroplast membranes with randomized particles can lead to a resegregation of specific types of intramembranous particles into the reformed stacked membrane regions, we explored the relationship between membrane stacking and particle segregation in greater detail. Two possible mechanisms of thylakoid membrane stacking may be envisaged: the membranes could gradually zip together as stacking particles from adjacent thylakoids move into the right position to crosslink or, alternatively, the membranes could

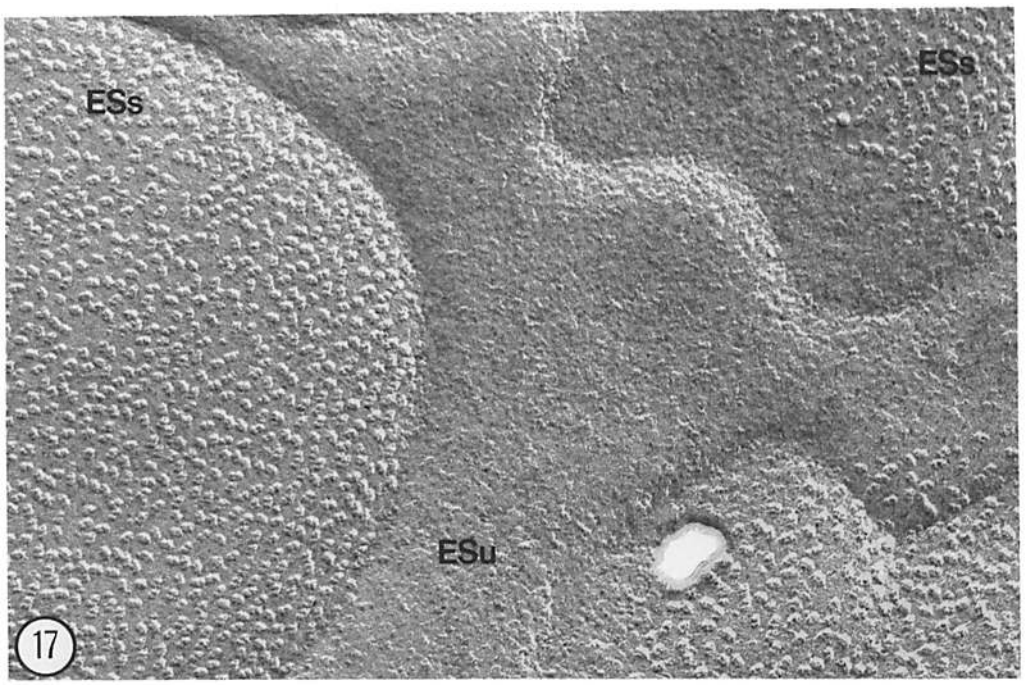


FIGURE 17 True inner ES-surface of a spinach thylakoid unstacked and then allowed to restack in vitro for 1 h in the presence of 5 mM $MgCl_2$ as described in Materials and Methods. The large particles containing subunits appear again aggregated in the stacked membrane regions (*ESs*), the unstacked areas (*ESu*) being essentially depleted of the particles. × 95,000.

first adhere randomly together, after which, the larger particles that diffuse into the stacked regions would become trapped there. These two theories can be distinguished by kinetic studies of the restacking process.

Experimentally unstacked thylakoids were allowed to restack in the presence of 2 and 5 mM $MgCl_2$ for periods of 5–60 min. To stop the stacking reaction at different time intervals, 0.5% glutaraldehyde was added to the restacking medium before the membranes were pelleted and further processed for freeze-fracture or deep-etch experiments. (In control experiments, glutaraldehyde fixation was shown to prevent stacking of experimentally unstacked membranes in the presence of Mg^{++} ions, and to prevent unstacking of stacked membranes in low ionic strength solutions. However, since glutaraldehyde fixation affects the number of particles that cleave with EFs and EFu faces, the experiments were also repeated without glutaraldehyde fixation, albeit with less timing accuracy.) Figs. 19 and 20 are freeze-fracture micrographs of experimentally unstacked thylakoids that were allowed to restack for 10 min in the presence of 2 mM $MgCl_2$. Fig. 19 depicts cross-fractured thylakoids possessing distinct regions of membrane stacking. Similar, roughly circular areas of membrane contact may be recognized on EF membrane faces (Fig. 20). Careful examination of Fig. 20 reveals, furthermore, that thylakoid membranes can become stacked in less than 10 min without significant segregation of the large EF particles into the stacked regions. This observation is consistent with the hypothesis that complete restacking of thylakoid membranes occurs in two steps: (*a*) a rapid adhesion of adjoining membrane surfaces with little particle movement; and (*b*) a slower diffusion of additional globular membrane components into the stacked regions where they become trapped by linking up with components in the adjacent membrane.

Further support for the two-step restacking hypothesis has come from restacking experiments with monovalent ions. While complete thylakoid membrane restacking in terms of particle segregation can be brought about within ~1 h by as little as 2 mM $MgCl_2$ (Fig. 16), similar structural changes can only be induced by monovalent ions when 40 times higher concentrations are used, i.e. >80 mM NaCl. As seen in Fig. 21, experimentally

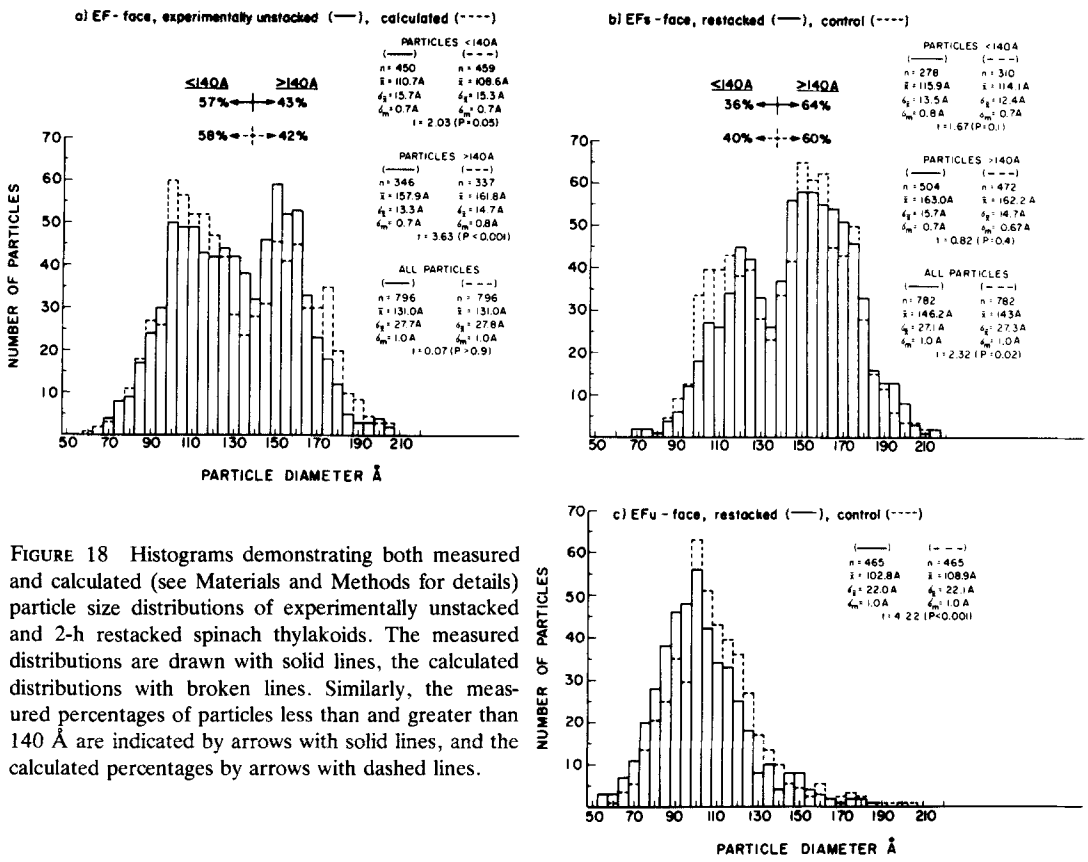


FIGURE 18 Histograms demonstrating both measured and calculated (see Materials and Methods for details) particle size distributions of experimentally unstacked and 2-h restacked spinach thylakoids. The measured distributions are drawn with solid lines, the calculated distributions with broken lines. Similarly, the measured percentages of particles less than and greater than 140 Å are indicated by arrows with solid lines, and the calculated percentages by arrows with dashed lines.

unstacked thylakoids suspended in a 50-mM NaCl solution for 1.5 h can carry out the first step of membrane stacking, i.e. they can stick together, but only a minimal amount of particle segregation can be detected. However, when the NaCl concentration is increased to >80 mM, more and more stacked membrane regions with higher EFs particle densities are observed (Fig. 22). Thus, aggregation of large particles into stacked membrane regions appears to require higher concentrations of monovalent ions than simple membrane adhesion.

Chl a:Chl b Ratios in Subchloroplast Fractions

We have measured chl a:chl b ratios of grana and stroma membrane fractions obtained by French press treatment of control, experimentally unstacked, and restacked chloroplasts to determine whether the movements of the intramembranous particles are paralleled by changes in the distribution of chlorophyll molecules between stacked and unstacked membrane regions. Corresponding changes might be expected if the particles contained sizable quantities of bound pigment molecules. The results, summarized in Table II, clearly establish such a relationship. The fact that the chl a:chl b ratios of the grana and stroma fractions of 10-min restacked thylakoids are already quite different, despite the minor changes in particle distribution (Fig. 20), could be related to the different processing of the samples. While Fig. 20 shows a chloroplast whose particle movements were stopped by glutaraldehyde fixation after 10 min of restacking, the corresponding grana and stroma fractions relate to samples passed after 10 min through a French press, which roughly separates stacked and unstacked membrane regions but which does not prevent further resegregation of the particles while the membranes are passed, during the next 10 min, three more times through

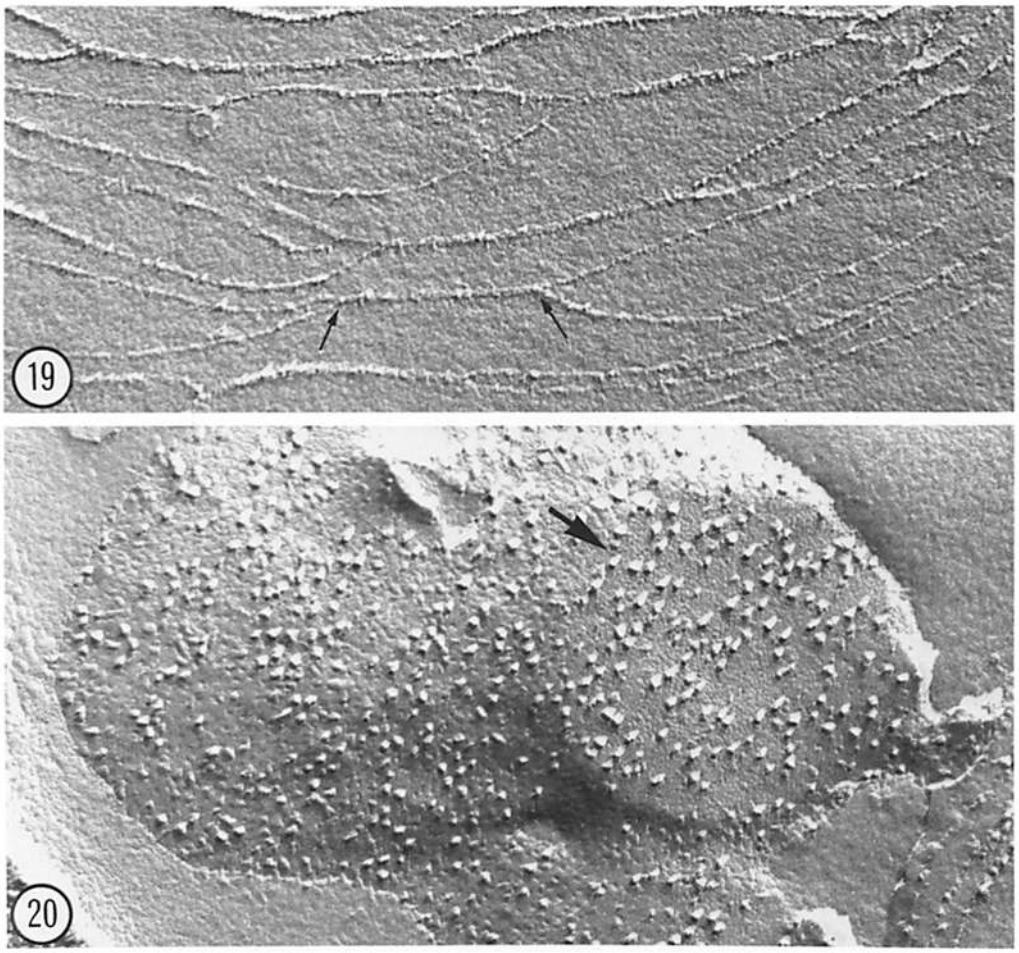


FIGURE 19 Isolated spinach thylakoid membranes unstacked and then allowed to restack for 10 min by the addition of 2 mM $MgCl_2$ (for further details see Materials and Methods). Where adjacent membranes come into contact (arrows), they appear normally fused. × 60,000.

FIGURE 20 EF fracture face of a spinach thylakoid unstacked and then allowed to partially restack for 10 min as described for Fig. 18. The flat, circular area of membrane contact (arrow) can be clearly distinguished from the more curved, noncontacted regions. Only a minor migration of large particles into the contact area (the "restacked" membrane region) appears to have occurred up to this point. × 95,000.

the press. Further complicating the issue is the fact that glutaraldehyde fixation reduces the number of particles that cleave with the EF faces by 10–50% (Fig. 20 was selected for illustrative purposes because it depicts a thylakoid with minimal reduction in EF particle density), while the centrifugation methods applied to separate fractions tend to select for membrane vesicles with different compositions. Although these differences in preparation of the samples preclude a quantitative correlation of particle movements and changes in chl a:chl b ratios, the parallel changes in both parameters upon unstacking and restacking of the membranes seem to strongly support the hypothesis of a close association of significant amounts of specific chlorophyll molecules with at least some of the particles. In this context it is interesting to note that the shifts in EFs and EFu particle sizes in experimentally restacked membranes, when compared with the controls (Fig. 18*b*, *c*), are also reflected in small but appropriate changes of the chl a:chl b ratios of the corresponding grana and

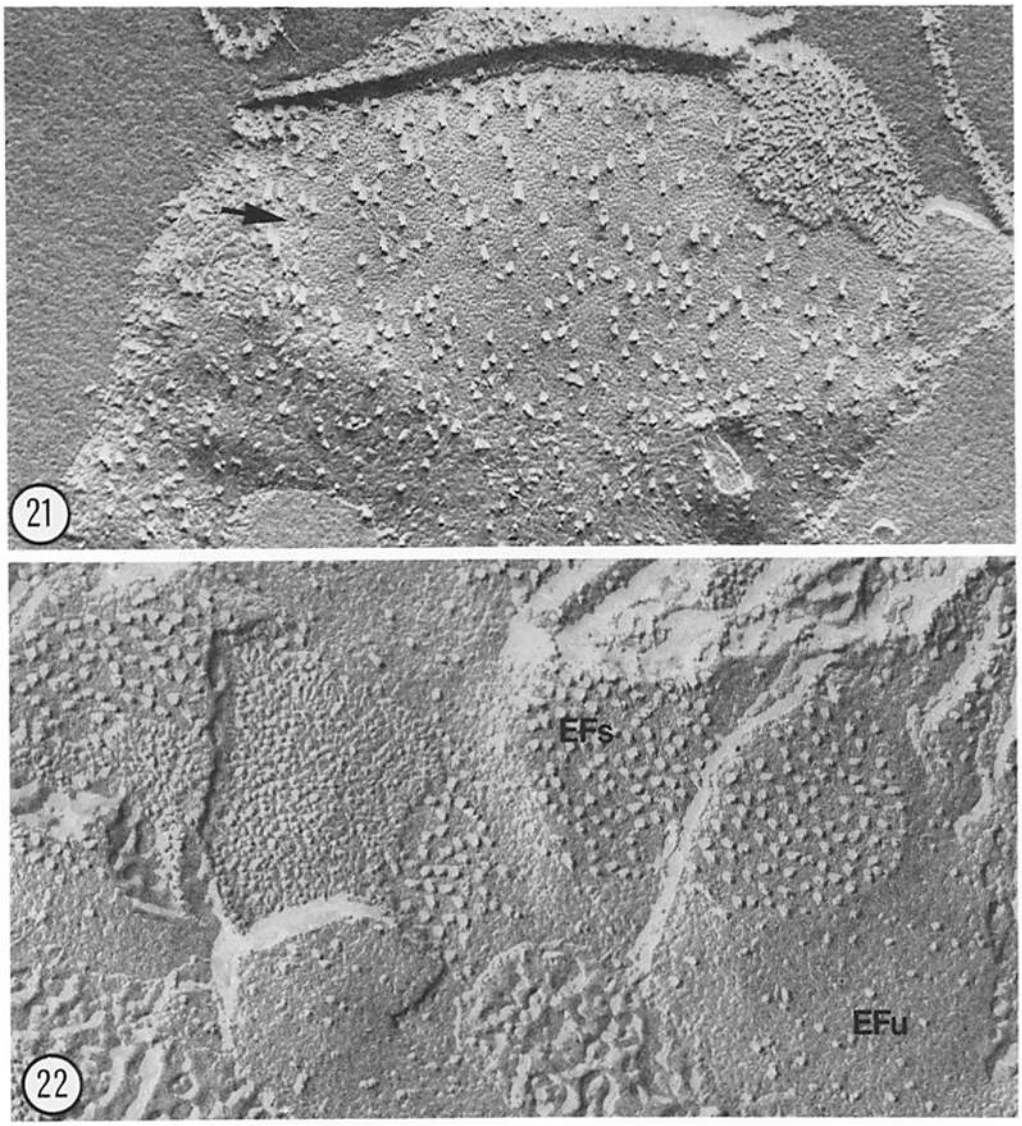


FIGURE 21 EF fracture face of a spinach thylakoid unstacked and then allowed to restack at room temperature for 1.5 h in the presence of 50 mM NaCl in a Tricine buffer (for details see Materials and Methods). The flat membrane area in the center of the micrograph (arrow) represents a contact region between two adjacent thylakoids (part of the upper thylakoid may be recognized near the top edge of the picture). It is surrounded by noncontacted membrane regions on the left and bottom sides. Note the rather insignificant segregation of large EF face particles into the membrane contact region even after 1.5 h of "restacking." × 83,000.

FIGURE 22 Freeze-fractured spinach thylakoids unstacked and then allowed to restack at room temperature for 1.5 h in the presence of 200 mM NaCl in a Tricine buffer. The EF faces show a clear segregation of the large particles between *EFs* and *EFu* regions. × 105,000.

stroma fractions (compare control and 2-h restacked samples in Table II).

TABLE II

Chl a:Chl b Ratios in Whole Chloroplasts and in Subchloroplast Fractions of French Press-Disrupted Pea Chloroplasts (Average of Five Measurements)

Chloroplast sample	Whole chloroplasts*	40K grana fraction*	144K stroma fraction*
Control	3.00 ± 0.05	2.50 ± 0.06	3.96 ± 0.13
Exp. unstacked		2.86 ± 0.07	
10 min restacked‡		2.74 ± 0.10	3.41 ± 0.34
30 min restacked‡		2.66 ± 0.09	3.85 ± 0.22
2 h restacked‡		2.41 ± 0.07	4.18 ± 0.12

* ± standard error.

‡ In 2mM $MgCl_2$, 50mM Tricine pH 7.6 at room temperature.

DISCUSSION

Structure-Function Correlations in Normal Thylakoid Membranes

Mühlethaler et al. (30) and Branton and Park (11) were the first to provide detailed descriptions of freeze-fractured spinach thylakoid membranes. However, it was left to Goodenough and Staehelin (18) to provide a coherent interpretation of all the fracture face images revealed by this preparation method. These authors demonstrated that wild-type thylakoid membranes of *Chlamydomonas reinhardtii* exhibited four qualitatively different fracture faces, two of which could be related to stacked (the complementary EFs and PFs faces) and two to unstacked (the complementary EFu and PFu faces) membrane regions (Fig. 1). Our present study has confirmed that the fracture face morphology of spinach thylakoids is essentially identical to that of *Chlamydomonas* thylakoids, and that in both systems stacked and unstacked membrane regions have a distinctly different organization within the plane of the membrane (Fig. 2).

In addition, our deep-etching micrographs have provided for the first time a clear correlation between particles seen on the EF face and those seen on the ES surface, the luminal surface of thylakoid membranes. Previous investigations of the luminal surface of thylakoid membranes (16, 27, 29, 34) have already revealed the presence of large particles containing two to four subunits, but—unlike in the present study—no clearcut relationship between these surface particles and the EF face particles was observed. While Park and Pfeifhofer (34) demonstrated that arrayed EFs and ESs particles had the same lattice parameters and similar dimensions, they were unable to provide a more general correlation of EF and ES particles because the washing procedures they applied before freezing of the deep-etch specimens led to the unstacking of the thylakoids, and consequently to the intermixing of the different kinds of membrane particles. We have found that these washing-induced changes of normal thylakoid membrane structure can be prevented by adding 2 mM $MgCl_2$ to the washing solutions. Under these conditions, the large ES particles composed of four subunits are nearly exclusively limited to stacked membrane regions (Fig. 6; 80–85% are located in stacked regions and 15–20% in unstacked ones), and their density and distribution correspond to the density and distribution of the EF face particles in stacked and unstacked membrane regions (Fig. 4, Table I). Thus, all EF particles seem to be related to the same membrane component although their average diameter differs considerably in EFs and EFu regions, respectively (Figs. 2–4). A possible interpretation of this phenomenon will be presented later.

The particle size histograms of spinach thylakoids prepared during the present study appear similar to those reported for *Chlamydomonas* (18, 32). However, possibly because of the finer measuring scale used, our spinach histograms demonstrate somewhat simpler but more distinct and more interpretable size distribution patterns than the published *Chlamydomonas* histograms. On the basis of these refined histograms, we can now distinguish four major categories of intramembranous particles, two of which are seen on the EF faces, and two on the PF faces (Fig. 3). As discussed earlier, comparison of EF face images with those of ES surfaces reveals that the two categories of EF particles (~115 Å and ~160 Å in diameter) contain identical ES elements, thus suggesting that the membrane component(s) giving rise to the EF particles can exist in at least two different "states."

Information pertaining to the possible origin of the two states has come from recent observations of Armond et al. (4) on chloroplasts isolated from pea plants grown for 7 days in the dark followed by 2 days in an intermittent light regime (ImL = 2 min light, 2 h dark). Such chloroplasts contain no light harvesting pigment-protein complexes (LHC, also known as chl a:b protein; 23, 44), no grana and no Mg^{++} regulation of "spillover" al-

though full photochemical activity is present (3, 12). Freeze-fracture examination of ImL thylakoids reveals a dramatic decrease in the size of the EF face particles to 80 Å. Transfer of the ImL plants to continuous white light results in the appearance of LHC in the chloroplast membranes, a concomitant increase in the size of the EF face particles, the formation of grana, and the aggregation of the enlarged EF face particles into the areas of grana stacking. Furthermore, it was found that the increase in the size of the particles occurred in discrete steps that could not be explained by a simple aggregation of 80 Å particles into larger units. A similar increase in EFs particle sizes from 80 Å to 120 Å has also been observed during the greening of *Euglena* etioplasts (33), while the EFs faces of a chl b-less barley mutant lacking LHC contain 120 Å particles but no 160 Å particles (K. R. Miller, personal communication). These results provide strong support for the notion that the different size categories of EF face particles could contain different amounts of bound LHC. The fact that all EF particles are reduced to one size in ImL chloroplasts, and increase to normal sizes when the plants are exposed to continuous light, also provides further support for the theory that all EF face particles are structurally and functionally related and that they contain identical cores.

As pointed out in the introductory paragraphs stroma membranes contain mostly PSI activity and minimal amounts of PS II together with a high chl a:chl b ratio, while grana fractions have both PS I and PS II activities and a low chl a:chl b ratio. For these reasons, it is generally assumed that PS I complexes are found in both grana and stroma membrane regions, whereas active PS II complexes are limited mostly to grana membrane regions.

Examination of the detergent PS I fraction in freeze-fracture replicas by Arntzen et al. (7) indicated that the stroma membrane fragments with PS I activity contain only the smaller type of intramembranous particles, while the detergent PS II/PS I fraction derived from grana membranes was enriched in large particles. These observations led to the suggestion that the small freeze-fracture particles are markers of PS I and the large particles of PS II. Similar conclusions were drawn by Sane et al. (37) after their structural examination of the stroma and grana fractions obtained by French pressure cell treatment. It has also been pointed out (1, 6, 7, 45) that the asymmetric distribution of the small and large particles between the two complementary fracture faces of thylakoids is consistent with biochemical and immunological sidedness studies of PS I and PS II complexes in the membrane, as well as with vectorial aspects of the photochemical reactions associated with the complexes.

The morphological findings presented in this paper have both confirmed and extended these correlations between fracture face particles and PS I and PS II complexes. To this end, the large EF particles, proposed to correspond to the PS II complexes, not only cleave with the luminal leaflet and bridge the width of the membrane (26, 28, 34) but also appear to contain identical cores and to protrude clearly from the luminal membrane surface. In addition, our observation of 80–85% of the EF particles being confined to grana membranes and 15–20% to stroma membranes correlates well with the finding that anti-PS II and anti-chl a antibodies can bring about a 15–20% reduction in PS II activity when allowed to react with isolated, normally stacked thylakoids (36, 45, 47). Apparently, only the PS II complexes associated with unstacked stroma membranes are accessible to the antibodies. The PF face particles, which are believed to correspond to the PS I complexes, both protrude slightly from the stromal surface (28) and cleave with the stroma membrane leaflet. In contrast to the EF particles, they are also fairly evenly distributed between stroma and grana membrane regions, similar to the distribution of PS I activities (8).

Fig. 23 represents an improved model of chloroplast membranes of higher plants, which illustrates how the present findings can be integrated with information developed in other studies of our laboratory (4, 18, 27, 28, 41)[1] as well as with results obtained by other workers (6, 7, 34, 37, 43, 45). Note the nonrandom distribution of all types of particles between stacked and unstacked membrane regions, and the asymmetric disposition of the particles with respect to the lipid bilayer continuum. The different particles have been drawn roughly to scale and are shown in approximately the observed proportions.

Membrane Changes Associated with Unstacking and Restacking of Thylakoids

The in vitro unstacking and restacking experiments reported in this paper have provided further

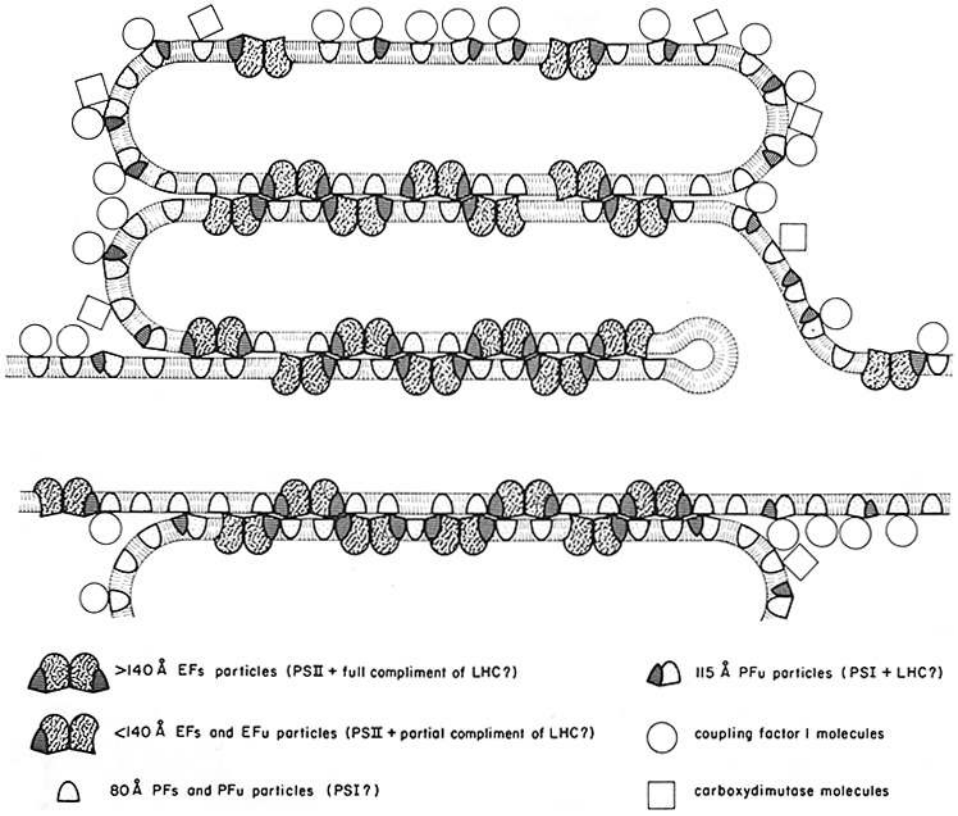


FIGURE 23 Improved model of chloroplast membranes illustrating how the present findings relate to our current understanding of thylakoid structure of higher plants and of green algae. Note the distribution of the different particle categories between stacked and unstacked membrane regions, the disposition of the particles with respect to the lipid bilayer continuum, and the spatial association of the particles in the stacked membrane regions. The light-harvesting complexes (*LHC*) have been arranged according to the suggestions of Armond et al. (4).

evidence for the structural relationship between the large EF face particles (Figs. 2 and 4) and the large, subunit-containing particles seen on ES surfaces (Fig. 6). They have provided unambiguous evidence for *reversible and directed* translational movements of integral proteins (39) of thylakoid membranes, and for parallel movements of chl a and chl b molecules. Finally, they have also allowed us to probe some of the parameters that govern these movements.

Experimental unstacking of *Chlamydomonas* thylakoid membranes (32) in a low-salt medium leads to complete intermixing of the particulate components of previously stacked and unstacked membrane regions. Ojakian and Satir (32) demonstrated this intermixing by statistically analyzing changes in the particle size histograms of the different fracture faces. In the present study we have shown that, because the large ESs particles have a distinct surface morphology that is not affected by the freeze-etching process, the spreading of this type of particle from stacked membrane regions to unstacked ones upon in vitro unstacking of the thylakoids can be monitored directly. Furthermore, since *all* EF face particles seem to be structurally related to the large ES particles, the movements of the ES particles may now be monitored directly on EF faces without the need for simultaneous particle size measurements.

The finding that unstacking- and restacking-induced movements of intramembranous particles are paralleled by changes in the distribution of chl a and chl b molecules between grana and stroma membrane regions (Table II) supports the hypothesis that sizable quantities of pigment molecules are bound to intramembranous particles. This re-

sult is consistent both with the observed relationship between EF particle size and LHC content of greening ImL thylakoids (4) and with the biochemical isolation of PS II-LHC complexes from chloroplast membranes (43).

One of the more unexpected results of our unstacking experiments with spinach chloroplasts is the change in size of a fraction of the EF face particles that accompanies the process. When the values of the measured and calculated EF face histograms of experimentally unstacked thylakoids (Fig. 18 *a*) are carefully analyzed, a statistically significant *increase* in the size of the <140-Å particles ($P = 0.05$) and a highly significant *decrease* in the diameter of particles >140 Å ($P < 0.001$) is observed. Because the shifts are in opposite directions and are of similar magnitude, the average size of all EF face particles remains constant. If the different size categories of EF face particles correspond, as suggested earlier, to PS II complexes with different amounts of bound LHC molecules, then the most likely explanation for our observation would seem to be that experimental unstacking (removal of ions) destabilizes the association between PS II core complexes and LHC molecules. As a result, the largest EF face particles with the greatest numbers of bound LHC molecules (each light-harvesting complex probably contains several LHC molecules; reference 4) would tend to lose some of these molecules to smaller EF face particles, which lack a full complement of bound LHC molecules. In other words, besides having a greater affinity for stacked membrane regions than their smaller counterparts, the large (>140 Å) EF particles appear to be stabilized by conditions that promote stacking. Experimental unstacking seems both to promote the exchange of LHC molecules between PS II complexes and to lead to a more even distribution of LHC molecules among these complexes.

Time-course studies of the restacking process show that it occurs in two steps: a rapid adhesion of adjoining membranes with little or no particle movement, and a slower diffusion of additional grana membrane components into the stacked regions where they become trapped by linking up with components of the adjacent membrane. At room temperature, the first step is concluded in approximately 10 min, while the second requires 1–2 h. The failure of Ojakian (32) to observe particle resegregation of restacked thylakoid membranes can probably be attributed to the fact that he did not allow sufficient time for the resegregation process to occur.

Light-induced changes in the distribution and size of EFs face particles have also been observed in isolated spinach chloroplasts suspended in a sodium acetate solution (44). Upon illumination, groups of EFs particles aggregate into small clusters in which the particle density increases from 2,000 to 3,400/μm^2. Simultaneously, the average diameter of the particles drops from 160 to 110 Å. Although these studies address themselves to a different type of membrane mobility than that described in the present communication, they, too, provide strong support for the dynamic nature of chloroplast membranes.

Particle Movements in Other Membrane Systems

Reversible translational movements of intramembranous particles have also been observed in erythrocyte ghosts suspended in media of different pH (35). Randomly dispersed particles are seen at neutral pH, while aggregation of the particles occurs at pH 5.5. In more recent studies, Elgsaeter and Branton (15) have determined that these pH-dependent particle movements can only be observed after partial but not complete removal of the water-soluble, filamentous membrane protein, spectrin. Similarly, the directed movement of cell surface antigenic sites associated with capping in lymphocytes (24, 42) is also dependent on filamentous cytoplasmic components (14, 46). In contrast, the reversible particle movements associated with the in vitro unstacking and restacking of isolated thylakoid membranes is independent of any filamentous stroma components, and seems to be controlled solely by the concentration of cations in the medium and by the establishment of regions of contact between adjacent membranes. In light of these findings, it will be interesting to determine whether the aggregation of the particulate elements of gap junctions during gap junction formation between embryonic or tissue culture cells (9, 13, 22) is dependent on functional cytoplasmic filaments or microtubules, or whether their assembly is governed by factors similar to those found to operate in the thylakoid system. That the formation of gap junctions could resemble the formation of stacked membrane regions in chloroplasts is suggested by the fact that in both systems the membrane-membrane interactions are me-

diated by divalent cations. Removal of these ions leads to inhibition of intercellular communication through gap junctions (25) and to unstacking of thylakoid membranes.

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