FREEZE-FRACTURE STUDIES ON BARLEY PLASTID MEMBRANES I. WILD-TYPE ETIOPLAST

by

DAVID J. SIMPSON

Department of Physiology, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen, Valby

Keywords: Envelope, isolation, perforated thylakoids, phototransformation, primary lamellae, prolamellar body, thylakoids, tubules

Etioplasts from wild-type barley seedlings have been isolated and their membranes examined by freeze-fracturing. All the membranes seen in thin sections of fixed etioplasts have been characterised in terms of the number of freeze-fracture particles per square micron, average particle size and size distribution. This was done for both fracture faces of the primary lamellae, the perforated thylakoids resulting from photodispersal of the prolamellar body, and for both membranes of the etioplast envelope. These membranes, which were identified by their morphology and relationship to one another, as known from thin section studies, could be readily distinguished from one another on the basis of their freeze-fracture ultrastructure. The structure of the prolamellar body has also been examined by freeze-fracturing and was consistent with the model proposed from thin section studies. Freeze-fracturing of the tubules formed by low temperature photodispersal of the prolamellar body revealed a particulate fine structure. The data obtained for the freeze-fracture ultrastructure of wild-type etioplast membranes will form the basis for comparison with that of the membranes from barley plastid mutants.

1. INTRODUCTION

Etioplasts are the plastids found in leaves of plants grown from seed in the dark. They represent an arrested stage in the normal development of proplastids into chloroplasts, and transform into chloroplasts after exposure to white light. Some other plastid steady-states

can be induced in the leaf by suitable light or temperature regimes, and many more by inducing plastid mutations. Such plastid mutants have proved valuable in studying pigment synthesis, photosynthesis and the development of chloroplast thylakoids. The plastids of many mutants resemble intermediate stages in the

development of chloroplasts from etioplasts during greening under continuous light and enable the reproducible isolation and investigation of a uniform population of such plastids (20). Plastid mutations in barley have been well characterised genetically, and much work has been done on their biochemical and photosynthetic capacities, and ultrastructure as revealed by thin sections of fixed material (20, 27).

Since the thylakoid membrane is the site of so many of the photosynthetic reactions of the plastid, attention has been focussed on its properties and structure. One technique that is particularly useful for studying thylakoid structure is freeze-fracturing, which is unique in its ability to permit the investigation of the structure of the hydrophobic region of chemically unaltered membranes. Fracturing at low temperature cleaves the lipid bilayer and reveals the topography of the membrane in this plane (1). There is mounting evidence that the particles seen in freeze-fracture replicas represent polypeptides organised into functional units (5). One of the aims of the project of which this study is a part is to attempt to correlate the polypeptide composition of plastid thylakoids with their freeze-fracture ultrastructure (11, 19).

There have been relatively few freeze-fracture investigations of etioplast membranes. The particle density of the primary lamellae of etioplasts of barley (16) and *Euglena* (14) have been measured, and the structure of the prolamellar body of bean (3, 4), *Euglena* (14, 15), *Chlorella* (17) and barley (16) has been described, but not to the extent of similar work on chloroplast membranes (22). The freeze-fracture appearance of the two membranes of the etioplast envelope has not been reported.

The present communication describes in detail the freeze-fracture appearance of the prolamellar body and characterises the primary lamellae and envelope membranes of barley etioplasts in terms of their freeze-fracture particle density and size distribution.

2. MATERIALS AND METHODS

2.1. Plant material

Wild-type barley (*Hordeum vulgare* cv. Svalöfs Bonus) was used since most of the plastid mutants have been induced in this cultivar (27). Seedlings were grown in plastic trays containing vermiculite moistened with tap water, for 6 days in the dark at 20°C.

2.2 Preparation of etioplasts

The apical 5-8 cm of etiolated seedlings were harvested in the dark, wrapped in aluminium foil to exclude light, and placed in ice for 1 h. The pre-cooled leaves were ground in 2-3 volumes of 0.6 M-glycerol, 0.1 M-tricine, pH 7.9, 0.003 M- $\text{Ca}(\text{NO}_3)_2$ or 0.4 M-sucrose, 0.05 M-sodium phosphate buffer, pH 7.3, 0.01 M-KCl, 0.002 M- MgCl_2 , using a modified kitchen homogeniser (13). [This and subsequent operations were carried out at 0°C in the light since the prolamellar body is stable to illumination at low temperature (9). Photodispersal of the prolamellar body, when desired, was induced by a brief exposure of the leaves to light at room temperature before wrapping in aluminium foil and placing on ice (10).] The resulting slurry was squeezed through a layer of 31 μm mesh nylon gauze and allowed to pass through a second layer of the same material without squeezing. After centrifugation for 5 min at 1,400 g, the yellow pellet was resuspended in a small volume of the grinding medium and an equal volume of 60% glycerol was added to give a final concentration of 30% glycerol. The resulting suspension was centrifuged for 5 min at 5,000 g and the resulting pellet used for freeze-fracturing.

2.3. Electron microscopy

Prior to the addition of 60% glycerol to the plastid pellet resuspended in grinding medium, this material was sampled for conventional electron microscopy. A small volume (about 0.2 ml) was injected with a Pasteur pipette into 10

Abbreviations: EF = endoplasmic fracture; IEF = inner envelope endoplasmic fracture; IPF = inner envelope protoplasmic fracture; OEF = outer envelope endoplasmic fracture; OPF = outer envelope protoplasmic fracture; PF = protoplasmic fracture face; S.D. = standard deviation.

ml of 2% glutaraldehyde in 0.05 M-phosphate buffer, pH 7.3. After a brief centrifugation the pellet was resuspended in fresh 2% buffered glutaraldehyde and fixed for 1 h at 0°C. The pellet was washed three times with phosphate buffer and postfixed with 1% OsO₄ at 0°C. After 1 h, the intact pellet could be gently dislodged from the bottom of the glass centrifuge tube, allowing the OsO₄ to diffuse into the 1 mm thick pellet from both sides for a further 1 h. The pellet remained intact during subsequent dehydration and infiltration and was cut into transverse slices immediately before embedding in SPURR's resin. Thin sections were cut with a diamond knife so as to extend from the top the bottom of the organelle pellet, picked up on a collodion/carbon coated 3 mm slotted copper grid and post-stained for 30 min at 60°C with 5% uranyl acetate, followed by 30 min at 20°C with REYNOLD's lead citrate in an automatic staining apparatus.

2.4. Freeze-fracturing

Unfixed etioplasts pelleted in 30% glycerol were frozen in Freon 22 cooled by liquid nitrogen. Replicas were made according to standard techniques on a Balzers BAF 301 freeze-etching machine (Balzers AG, Liechtenstein). Specimens were fractured at -110°C and replicas cleaned for 2 h in concentrated sodium hypochlorite, overnight in 10-20% chromic acid and washed four times in glass distilled water. Replicas were picked up on 200 mesh copper grids made hydrophilic by treating with a 1% solution of polybutylene in xylene, and examined with a Siemens Elmiskop 102 operated at 80kV with a 30 µm objective aperture. All freeze-fracture micrographs are printed so that the direction of shadowing is towards the top of the page. The terminology used in that of BRANTON et al. (2) by which the two freeze-fracture faces of any membrane can be designated protoplasmic (PF) or endoplasmic (EF).

2.5. Measurements and calculations

The electron microscope was adjusted to standard conditions by focussing the second in-

termediate lens in the plane of the selected area aperture before taking a series of micrographs. Prior to taking each exposure, all lenses were normalised by reversing their polarity to remove hysteresis effects, and astigmatism was corrected at 100,000 × magnification using a television viewing screen (additional 9 × magnification). The microscope was calibrated at 50,000 × and 100,000 × nominal magnification with catalase crystals (Balzers Union) using a lattice spacing of 87.5 Å. This procedure was repeated at regular intervals and it was found that the magnification varied by less than 2% over a one year period when the microscope was operated as described above.

The particle densities (i.e., the numbers of particles per square micron) were measured on at least four micrographs of large flat membrane regions taken at 50,000 × and enlarged to 200,000 ×. A transparent plastic 20 × 20 cm grid subdivided into 100 2 × 2 cm squares (each corresponding to an area of 0.01 µm² at 200,000 ×) was overlaid on the micrograph and all particles counted within each square (22).

Particle sizes were determined by taking micrographs at 100,000 ×, enlarging them to 800,000 × and measuring the particles with a Hewlett-Packard HP-9864A digitiser interfaced with a HP-9825A computer, a HP-9871A printer and a HP-9872A plotter. The particle width was calculated by measuring the width of the shadow at the boundary between the opaque half of the particle and its shadow (22) using the digitiser cursor. There was, nevertheless, a degree of subjectivity in determining particle boundaries when measuring particles widths, especially for fracture faces with a high particle density. Whenever possible, all particles were measured within a contiguous area to avoid sampling bias. When particles were too close together to discern individual edges, they were not measured. The digitised values were converted into Ångström units by the computer program, which calculated mean particle size and standard deviation and plotted the data as a histogram. A separate histogram was plotted after every 100 or 200 measurements to monitor random variations in particle size from one micrograph to the next of the same fracture face for each membrane. The mean of these

sub-sets was calculated and varied less than 5 Å from the final average value calculated for all particles for each fracture face.

3. RESULTS

Etioplasts are readily distinguished by the presence of the pseudocrystalline prolamellar body within a double membrane envelope (6, 7, 26). By fixing and sectioning a representative sample it was shown that the isolation procedure was capable of producing a relatively pure preparation of intact etioplasts. Figure 1 shows a field of isolated etioplasts and indicates a low degree of contamination by other organelles and membranes. The intactness of the two membranes comprising the etioplast envelope is demonstrated in Figure 2, which also illustrates the two types of internal membranes of etioplasts – those of the prolamellar body and the primary lamellae. A higher magnification micrograph of a prolamellar body sectioned as to display the regular lattice planes of this hexagonally symmetrical structure is given in Figure 3. It is this kind of prolamellar body, which is the most common of the pseudocrystalline types, that has been examined by freeze-fracturing in this paper.

It was found that the isolation of etioplasts in a buffer containing glycerol and subsequent slow infiltration with glycerol to a final concentration of 30% caused vesiculation of the primary lamellae (Figure 4), although the other membranes did not seem to be affected. Consequently etioplasts were usually isolated in the sucrose-containing buffer and rapidly infiltrated with glycerol, pelleted and frozen soon after. This resulted in improved stability of the primary lamellae (e.g. Figure 24). It was essen-

tial when fixing samples for thin section electron microscopy to remove as much of the osmoticum as possible to avoid the artifacts associated with the reaction between OsO_4 and residual glycerol or sucrose (see Figure 1 of ref. 11).

3.1. The prolamellar body

In the majority of cases the prolamellar body cross-fractured to reveal an irregular convoluted structure yielding little information about the internal structure. When the plane of cross-fracturing occasionally coincided with a natural lattice plane, a regular array of cross-fractured tubes was seen (Figure 5). In a few cases the fracture plane partially cleaved the membranes through a lattice plane, revealing a regular hexagonal pattern of projections (Figure 6). The average separation of such projections was $520 \pm 6 \text{ Å}$ ($\pm \text{S.D.}$), so they probably correspond to the tubules seen in thin sections, which had a centre-to-centre distance of $502 \pm 48 \text{ Å}$ ($\pm \text{S.D.}$).

In Figure 7 the membranes of the prolamellar body have cleaved to reveal tetrahedrally branched tubules, corresponding to similar structures seen in thin section (Figure 3). Most of the tubules project out of the plane of the page, but occasionally the fracture plane has gone below the lattice plane, producing holes. These can also be seen in Figures 9 and 11, where the fracture plane has followed a different lattice plane. It is not known to what feature of the prolamellar body – as it is seen in thin sections – the regularly spaced projections in Figure 11 correspond. They are smaller than most tubules but may correspond to those seen in rows in thin section (Figure 10, arrows). In Figure 12 rows of projecting tubular elements

Figure 1. Representative field of etioplasts isolated in sucrose medium showing intact envelopes and an acceptably low degree of contamination by other organelles and membranes. $\times 9,000$ (Bar = $2\mu\text{m}$)

Figure 2. An isolated etioplasts (sucrose medium) showing intact envelope membranes (E), prolamellar bodies (PLB) and primary lamellae (PL). $\times 33,000$ (Bar = $1.0\mu\text{m}$)

Figure 3. Higher magnification micrograph showing the fine structure of the prolamellar body. This structure is considered to consist of interconnected tetrahedrally branched tubes. Note the presence of dark-staining particles, the »ribosome-like particles«, within the stroma spaces enclosed by the tubes. $\times 98,800$ (Bar = $0.5\mu\text{m}$)

Figure 4. Freeze-fractured intact etioplast isolated in glycerol medium and not fixed. Note the distinctive appearance of the prolamellar body (PLB). Both envelope membranes can be seen surrounding the etioplast (arrows) and the membranes of the primary lamellae (PL) have broken up into numerous vesicles (V). $\times 35,300$ (Bar = $1.0 \mu\text{m}$)

alternate with cross-fractured stroma space enclosed by the tubule network. This micrograph shows the relationship between many of the freeze-fracture structures seen in Figures 7, 8, 9 and 11 and should be compared with the thin section images of Figures 3 and 10.

3.2. Photodispersal of the prolamellar body

There were indications from some of the freeze-fractured prolamellar body images that

the tubular membranes contained intramembranous particles (e.g., Figures 8, 9, and 11). In most instances, however, the membranes cross-fractured, and when they were cleaved it was not possible to make quantitative measurements on the highly convoluted surfaces. Accordingly, the prolamellar body was dispersed by a brief exposure to light at room temperature followed by gradual cooling in the dark to 0°C (10). This resulted in the formation of perforated thylakoids, often in concentric

Figure 5. Prolamellar body that has cross-fractured to reveal a regular pattern. This image may be interpreted as consisting of cross-fractured stroma spaces, each such area being surrounded by three holes (asterisks), corresponding to the vertical arm of a tetrahedrally branched tubular subunit extending perpendicular to and below the plane of the page. $\times 237,000$ (Bar = $0.1 \mu\text{M}$)

Figure 6. Freeze-fractured prolamellar body showing a regular hexagonal array of protruding tubes. Three of these (asterisks) surround a stroma space in what might be considered to be the complementary image of Figure 5. $\times 315,000$ (Bar = $0.1 \mu\text{m}$)

Figure 7. As in Figure 6, except that the fracture plane has cleaved the tube membranes, revealing the connections (arrows) between protruding tubes (cf. Figure 3). In some places the fracture plane has passed under the tubes, forming holes (arrowheads). $\times 237,000$ (Bar = $0.1 \mu\text{m}$)

Figure 8. Prolamellar body fractured to reveal a linear interconnection (arrow) between two protruding tubes, in contrast to those in Figure 7. $\times 237,000$ (Bar = $0.1 \mu\text{m}$)

Figure 9. Freeze-fractured prolamellar body revealing several characteristics, including tube holes (arrows), protruding tubes (asterisks) and smaller, regularly spaced particles (circled) like those of Figure 11. There are other particles (arrowheads) which are irregularly spaced and smaller still and resemble the normal freeze-fracture particles of membranes. $\times 237,000$ (Bar = $0.1 \mu\text{m}$)

Figure 10. Thin section of a prolamellar body through a different lattice plane to that in Figure 3, possibly the same as that fractured in Figure 8. $\times 97,800$ (Bar = $0.2 \mu\text{m}$)

Figure 11. Prolamellar body fractured through a different lattice plane to that in Figure 7. The protruberances (circled) are smaller than those in Figure 7, and may correspond to the rows of tubules seen in Figure 10. $\times 237,000$ (Bar = $0.1 \mu\text{m}$)

Figure 12. Freeze-fractured prolamellar body showing the cross-fractured stroma spaces (asterisks) surrounded by three protruding tubes (arrows) and, between them, three smaller protruding structures (circled) that may correspond to similar structures seen in Figures 9 and 11. If so, they are clearly not within the stroma space and could not correspond to the ribosome-like particles. $\times 237,000$ (Bar = $0.1 \mu\text{m}$)

shells (Figure 13). These membranes are referred to as photodispersed lamellae and are considered to be derived from the membranes of the prolamellar body. They exhibited normal membrane splitting when freeze-fractured, and it was therefore possible to make measurements of their freeze-fracture particle density and size.

The appearance of these membranes when cross-fractured is shown in Figure 14. Upon cleavage, they revealed two different fracture faces (Figure 15). Since these thylakoid membranes enclose an endoplasmic space, it follows from their morphology that the fracture face with circular depressions and low particle density is the EF face, and the fracture face with the raised circular regions and higher particle

density is the complementary PF face. It can be seen by visual inspection and by referring to Table I, that the EF face contained much fewer freeze-fracture particles per square micron than the PF face, although the average particle size was similar (Table II and Figure 27). Evidence that depressions and raised areas correspond to perforations of the membrane can be seen in transverse thin sections (Figure 13) and cross-fractured membranes (Figure 14). Particles were never seen in these regions, and measurements of particle density did not correct for the area of the perforations.

Many of the photodispersed etioplasts contained straight, tubular structures that resulted from the disintegration of the prolamellar body. These are shown in thin section in Figure 16,

Table I

Freeze-fracture particle densities for wild-type barley etioplast membranes.
S.E. = standard error.

Membrane	Face	No./ $\mu\text{m}^2 \pm \text{S.E.}$	Area measured (μm^2)
Primary	EF	380 ± 13	2.07
	PF	4555 ± 43	0.83
Photodispersed	EF	188 ± 9	2.40
	PF	2614 ± 38	1.43
Envelopes			
	Outer		
	EF	766 ± 14	4.43
	PF	1600 ± 20	2.60
	Inner		
	EF	2390 ± 43	1.32
	PF	7492 ± 73	0.52

where they appear to be continuous with the primary lamellae. The corresponding freeze-fracture image appears in Figure 17, in which straight tubules seem to be in contact with the photodispersed prolamellar body and perforated lamellae. In thin sections, the tubules were hollow when transversely sectioned, with an electron translucent central lumen $65 \text{ \AA}$ in diameter, surrounded by darker staining walls (Figures 18 and 19). In longitudinal sections (Figure 18) these structures had parallel sides with a diameter of $195 \pm 9 \text{ \AA}$ ($\pm \text{S.D.}$).

Freeze-fractured photodispersed etioplasts contained straight-sided cylinders (Figures 17, 20 and 21) with a diameter of $198 \pm 11 \text{ \AA}$ ($\pm \text{S.D.}$), which is in good agreement with the value obtained from thin sections of the tubules formed by prolamellar body photodispersal. When fractured longitudinally to produce con-

cave surfaces, these structures revealed a regular pattern of fracture particles (Figure 20) giving the appearance of striations with a centre-to-centre distance of $77 \pm 2.4 \text{ \AA}$ ($\pm \text{S.D.}$), oriented at an angle of 118° to the long axis of the tubule. If the tubules are considered to enclose endoplasmic space (as are the tetrahedrally branched tubules of the non-dispersed prolamellar body), then the striated concave surface is the PF face. Freeze-fracture particles could not be resolved on the convex EF face (Figure 20). A tubule which had fractured to produce both the concave PF and convex EF faces is shown in Figure 21.

3.3. Primary lamellae

The etioplast primary lamellae, which are thylakoids organised in approximately parallel lamellae extending from the prolamellar body

Table II

Freeze-fracture particle sizes for wild-type barley etioplast membranes.
S.D = standard deviation.

Membrane	Face	Average size ($\text{\AA}$)	S.D. ($\text{\AA}$)	No. counted
Primary	EF	85.7	17.6	500
	PF	70.4	14.8	800
Photodispersed	EF	85.8	14.3	240
	PF	78.0	17.4	800
Envelopes				
	Outer			
	EF	82.4	18.1	700
	PF	68.4	16.3	800
	Inner			
	EF	67.1	14.7	700
	PF	60.1	13.3	800

Figure 13. Transverse thin section through the perforated thylakoids resulting from photodispersal of the prolamellar body. Arrows indicate pores through the membrane. $\times 70,000$ (Bar = 0.5 μm)

Figure 14. Freeze-fracture image of the same type of membrane shown in Figure 13. Note the presence of perforations through the cross-fractured regions of the membranes (arrows) and concave and convex freeze-fracture faces on either side of the pores. $\times 84,000$ (Bar = 0.5 μm)

Figure 15. Freeze-fractured perforated thylakoids showing both fracture faces (EF and PF). The EF face is sparsely populated with freeze-fracture particles, and has numerous small circular depressions (asterisks), corresponding to the perforations seen in Figure 14. The PF face has a higher density of freeze-fracture particles and raised areas corresponding in size and number to the depressions seen on the EF face. $\times 296,000$ (Bar = 0.1 μm)

Figure 16. Thin section of isolated etioplast from leaves exposed to light at low temperature (0°C). Note the presence of straight tubules (T) which appear to be continuous with the membranes of the primary lamellae (arrows). $\times 65,000$ (Bar = 0.5 μm)

Figure 17. Freeze-fracture appearance of the same material as that shown in Figure 16. Note the presence of straight tubules (T) apparently connecting perforated thylakoids (arrow) and the remains of the prolamellar body (PLB). $\times 65,000$ (Bar = 0.5 μm)

Figure 18. Higher magnification micrograph of transversely and longitudinally sectioned tubules from etioplasts transformed at low temperature. $\times 124,000$ (Bar = 0.2 μm)

Figure 19. Transverse section of tubules at higher magnification $\times 237,000$ (Bar = 0.1 μm)

Figure 20. Freeze-fractured tubules showing a striated fine structure on the concave PF face and a smooth convex EF face. $\times 237,000$ (Bar = 0.1 μm)

Figure 21. Micrograph showing both the concave PF and convex EF face of the same tubule. $\times 237,000$ (Bar = 0.1 μm)

(Figure 2), usually cross-fractured when freeze-fractured, or cleaved for relatively short distances before cross-fracturing (Figure 22 and 24). It was relatively rare to see flat horizontal areas of freeze-fractured primary lamellae such as is shown in Figure 23. The EF face contained a lower number of particles per square micron than the PF face, although both faces of primary lamellae were characterised by a higher particle density than the corresponding faces of the perforated thylakoids resulting from photodispersal of the prolamellar body (Table I). The average particle diameter for each face is given in Table II and the particle size distribution is presented in histogram form in Figure 27.

3.4. Envelope membranes

An intact etioplast which has fractured to reveal the EF and PF faces of the primary lamellae is shown in Figure 24. It can be seen that one of the fracture faces of the envelope membranes has also been cleaved. In order to establish unequivocally that the membranes examined were those of the etioplast envelope, only those freeze-fracture images in which the fracture plane passed through both membranes of the envelope were used for measurement. In addition, only circular or elliptical areas with diameters greater than 3-4 microns were selected to avoid confusion with mitochondria, with the additional proviso that the fracture faces of the two membranes were both concave

Figure 22. Freeze-fracture image of primary lamellae (EF and PF faces), demonstrating their connection to the prolamellar body (PLB) and typical fracturing behaviour $\times 72,000$ (Bar = $0.5 \mu\text{m}$)

Figure 23. High magnification micrograph of the rarely seen flat horizontal areas of the primary lamellae, showing both EF and PF fracture faces. $\times 197,000$ (Bar = $0.1 \mu\text{m}$)

Figure 24. Freeze-fractured etioplast showing the relationship between primary lamellae (EF and PF faces) and the etioplast envelope, identified as the EF face of the outer envelope (OEF). $\times 58,000$ (Bar = $1.0 \mu\text{m}$)

or convex and close together, in order to rule out the possibility of confusing etioplast envelope membranes with two adjacent membranes from another source. Nuclear envelope

double membranes were rarely seen, and could be readily identified by the presence of pores. By means of these selection criteria it was also possible to determine which fracture faces

Figure 25. Freeze-fractured etioplast envelope, revealing both membranes. Since these faces are both convex, it follows that the uppermost face is the EF face of the outer envelope (OEF), and the one beneath it with numerous freeze-fracture particles is the PF face of the inner envelope. Only images of this type were used for measurements. $\times 113,000$ (Bar = $0.2 \mu\text{m}$)

Figure 26. The complementary image to that shown in Figure 25, showing two concave envelope membranes, identified by their relationship to one another as the EF face of the inner envelope (IEF) and the PF face of the outer envelope (OPF). Note that the IEF face has a higher freeze-fracture particle density than the OPF. $\times 113,000$ (Bar = $0.2 \mu\text{m}$)

Figure 27. Computer-drawn histogram plots of the size distributions of the freeze-fracture particles of etioplast internal membranes. The data have been sorted into 2.5 Å classes, a moving average calculated over 10 Å , and then plotted in 5 Å classes, as described in Material and Methods (2.5). Values for average size and standard deviation are given in Table II.

Figure 28. Computer-drawn histogram plots of the size distributions of the four freeze-fracture faces of etioplast envelope membranes. Average particle size and standard deviation are presented in Table II.

resulted from which envelope membrane by virtue of their relation to one another and to the stroma of the etioplast.

Figure 25 shows the convex fracture images of the EF face of the outer envelope membrane (designated OEF), which contained relatively few freeze-fracture particles, and the PF face of the inner envelope membrane (designated IPF), which was densely covered with freeze-fracture

particles. The complementary concave fracture faces of the outer and inner envelope membranes are shown in Figure 26. These are the outer PF (OPF) and inner EF (IEF) faces. The numbers of particles per square micron, and the average particle size are given in Tables I and II. The particle size distributions are shown in histogram form in Figure 28.

4. DISCUSSION

The three-dimensional organisation of the tubular elements (25) in the prolamellar body has been determined from thin sections of chemically fixed and embedded material. GUNNING and GUNNING & STEER (6, 7) suggested that many of the different types of prolamellar bodies could be considered to be constructed from tetrahedrally branched tubes interconnected in different ways. This model implies a high degree of membrane convolution and consequently predicts that a fracture plane inevitably includes cross-fractured tubes even when following a lattice plane. This was found to be the case for the majority of the freeze-fractured prolamellar bodies. Since the fracture plane follows the path of least resistance (lowest energy requirement), most prolamellar bodies cross-fractured irregularly and even when the fracture plane coincided with a lattice plane, it was extremely rare for the membranes to be cleaved, as they are in Figure 7. This type of freeze-fracture image is, however, entirely consistent with the model proposed by GUNNING (6) and does not support the model of parallel continuous undulating sheets with protrusions and depressions proposed by BROCHART (3, 4) for bean prolamellar bodies and by OPHIR and BEN-SHAUL (14, 15) for *Euglena*. It is not certain whether all of the different freeze-fracture images of barley etioplast prolamellar bodies can be explained in terms of GUNNING's model, but it does not seem necessary to invoke a new one.

Photodispersal of the prolamellar body into perforated thylakoids (also called perforated primary lamellae) is rapid and can occur in the dark at room temperature within 2 min of a brief illumination with white light (10). These membranes can persist for at least the first two hours of greening (7), corresponding to the lag phase for chlorophyll synthesis. The speed with which the membranes form, and the concomitant disappearance of the prolamellar body indicate that the perforated lamellae are formed by rearrangement of the membranes of the prolamellar body (26). Based on this assumption, characteristics of the freeze-fractured membranes of the prolamellar body can be deduced by examining the perforated

thylakoids. These membranes have a lower particle density on both EF and PF faces than the unperforated primary lamellae, although the two types of membranes are continuous with one another. This may be evidence that both types of membranes are precursors of the photosynthetic lamellae of chloroplasts, although most of the membrane material would originate from the prolamellar body.

The existence of straight tubules in etioplasts of dark-grown leaves has been known for some time (26), and their formation from the prolamellar body can be induced in isolated intact oat etioplasts by exposure to light for as little as 5 min (24). WELLBURN & WELLBURN consider that the process of prolamellar body photodispersal has been slowed down at 4°C, allowing the observation of a transient stage of straight tubules not normally seen when the process takes place at room temperature. These tubules are then proposed to fuse, forming perforated thylakoids which subsequently lose their perforations. The values of tubule dimensions obtained from thin sections of barley etioplasts are reasonably close to those given for oat etioplasts (24), namely an outside diameter of 250-280 Å and a central lumen of diameter 70-78 Å. Different stages in the photodispersal of barley prolamellar bodies were seen in the preparations used in the present study, presumably because there was a range of etioplast age and photosensitivity since most of the length of the first foliar seedling leaf was used (cf. 18).

The straight tubules reveal additional fine structural information when they were freeze-fractured, although this could not be seen in thin sections of the same material (see Figures 19 and 20). It is interesting in this respect that VON WETTSTEIN et al. (27) have induced the formation of tubules with a similar appearance to those reported here, by feeding delta-amino-levulinic acid in the dark to leaves of the mutants *xantha* -*f*²⁶, -*g*⁴⁵ and -*h*³⁵. They were able to discern a substructure in the walls of transversely sectioned tubules. HENNINGSEN (9) has proposed that a conformational change in the holochrome protein of phototransformed prolamellar body tubules is related to the dispersal of tubules into perforated lamellae. This step is

associated with the Shibata shift and dispersal of the prolamellar body and is inhibited at 0°C, which may explain why the straight tubules accumulate under these conditions. Furthermore, KAHN (12) has demonstrated regularly arrayed macromolecules in the walls of isolated bean prolamellar body tubules by negative staining. The centre-to-centre spacing of 80 Å measured for these particles is consistent with the molecular weight of 600,000 daltons estimated for the protochlorophyllide holochrome of bean leaves, and is remarkably close to the value of 77 Å found for the spacing of the PF particles of freeze-fractured photodispersed tubules. It is tempting to speculate that these PF particles correspond to those seen in the negatively stained preparations of KAHN and may be important in determining the structural configuration of the prolamellar body and photodispersed tubules.

The unperforated membranes of the primary lamellae are continuous with both the prolamellar body and the perforated thylakoids that it transforms into. The major differences in the freeze-fracture appearance of these two thylakoids, apart from the perforations themselves, are in the numbers of freeze-fracture particles per square micron (Table I). There are also measurable differences in both average particle size, and particle size distribution (Table II and Figure 27). Considerable care, however, needs to be exercised when making and interpreting these sorts of measurements. The digitiser error of 0.01 inch corresponds to 3.18 Å at a final magnification of 800,000. This is acceptable in terms of the resolution of the microscope, and the shadowing technique, but can lead to artifacts when plotting a histogram in 5 Å class widths. It was found that there were significant differences in the appearance of the histogram when the same data were replotted after simply shifting the class end points by 2.5 Å. This was overcome by calculating a moving average over a 10 Å interval for the data and then plotting in 5 Å wide classes. All histograms presented here have been smoothed in this way. When this is done, the distribution of particle sized for each of the fracture faces of etioplast membranes fits a normal distribution curve (Figures 27 and 28).

The lower particle density of the outer envelope membrane when compared with the inner membrane (Table I) is consistent with the hypothesis that the outer membrane is unspecifically permeable to low molecular weight metabolites, while the inner membrane is the site of specific metabolite transport (8). This correlation between freeze-fracture particle frequency and membrane metabolic activity has been pointed out for spinach chloroplast envelope membranes (21). The freeze-fracture appearance of inner and outer envelope membranes differs from one another and from that of the internal etioplast membranes. There are also measurable size differences in the freeze-fracture particles themselves (Table II, Figure 28). The particles of the inner envelope are smaller on average than those of the corresponding faces of the outer envelope, and fall within a narrower size range. The particles of the EF faces of inner and outer envelope membranes have a larger average size than the PF face of the same membrane, as well as a slightly larger size range. Both envelope membranes are completely different from internal membranes with respect to their freeze-fracture appearance. If the inner envelope membrane invaginates to give rise to internal membranes, the present results indicate that extensive changes in membrane organization must occur.

Characterisation of all of the membranes of wild-type etioplast by freeze-fracturing, in conjunction with those of the wild-type chloroplast (SIMPSON, in preparation), forms the basis for the comparison of the ultrastructure of the membranes of barley plastid mutants. The fact that the different types of wild-type etioplast membranes can be distinguished from one another solely on the basis of their freeze-fracture appearance (i.e., the number of particles per square micron) should prove useful in identifying etioplast membranes isolated for biochemical studies (see 21), or for freeze-etch characterisation of the membrane surfaces.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the contribution of professor DITER VON WETTSTEIN in suggesting this project and critically reviewing

the manuscript. I am grateful to cand. stat. LISBETH SEJERSEN and JØRGEN PEETZ for writing computer programs for measurement, statistical analysis and plotting of the data. I thank ANN-SOFI STEINHOLTZ, JEAN SAGE and NINA RASMUSSEN for their skilful technical assistance. Financial support was provided by the Commission of European Communities contract 029-76-ES DK solar energy program and by US PHS, National Institutes of Health (GM-22051).

REFERENCES

- BRANTON, D.: Fracture faces of frozen membranes. *Proc. Nat. Acad. Sci. USA* 55, 1048-1056 (1966)
- BRANTON, D., S. BULLIVANT, N. B. GILULA, M. J. KARNOVSKY, H. MOOR, K. MÜHLETHALER, D. H. NORTHCOTE, L. PACKER, B. SATIR, P. SATIR, V. SPETH, L. A. STAEHELIN, R. L. STEERE & R. S. WEINSTEIN: Freeze-etching nomenclature. *Science* 190, 54-56 (1975)
- BRONCHART, R.: Une nouvelle interprétation de la structure du corps prolamellaire basée sur des observations en cryodécapage. *C.r. Acad. Sci. Paris* 270, 1789-1791 (1970)
- BRONCHART, R.: Morphology of the prolamellar body as revealed by freeze-etching. VII Cong. Internat. Microsc. Electr. Grenoble Vol. III, 187-188 (1970)
- FISHER, K. A. & W. STOECKENIUS: Freeze-fracture purple membrane particles: protein content. *Science* 197, 4653-4657 (1977)
- GUNNING, B. E. S.: The greening process of plastids. I. The prolamellar body. *Protoplasma* 60, 111-130 (1965)
- GUNNING, B. E. S. & M. W. STEER: *Ultrastructure and the Biology of Plant Cells*. Edward Arnold, London (1975)
- HELDT, H. W. & F. SAUER: The inner membrane of the chloroplast envelope as the site of specific metabolite transport. *Biochim. Biophys. Acta* 234, 83-91 (1971)
- HENNINGSEN, K. W.: Macromolecular physiology of plastids VI. Changes in membrane structure associated with shifts in the absorption maxima of the chlorophyllous pigments. *J. Cell Sci.* 7, 587-621 (1970)
- HENNINGSEN, K. W. & J. E. BOYNTON: Macromolecular physiology of plastids VII. The effect of a brief illumination on plastids of dark-grown barley leaves. *J. Cell Sci.* 5, 757-793 (1969)
- HØYER-HANSEN, G. & D. J. SIMPSON: Changes in the polypeptide composition of internal membranes of barley plastids during greening. *Carlsberg Res. Commun.* 42, 379-389 (1977)
- KAHN, A.: Developmental physiology of bean leaf plastids II. Negative contrast electron microscopy of tubular membranes in prolamellar bodies. *Plant Physiol.* 43, 1769-1780 (1968)
- KANNANGARA, C. G., S. P. GOUGH, B. HANSEN, J. N. RASMUSSEN & D. J. SIMPSON: A homogenizer with replaceable razor blades for bulk isolation of active barley plastids. *Carlsberg Res. Commun.* 42, 431-439 (1977)
- OPHIR, I. & Y. BEN-SHAUL: Structural organization of developing chloroplasts in *Euglena*. *Protoplasma* 80, 109-127 (1974)
- OPHIR, I., A. TALMON & Y. BEN-SHAUL: A structural study of developing chloroplasts in *Euglena*. VIII Internat. Cong. Electron Microsc. Canberra Vol. II, 364-365 (1974)
- PHUNG NHU HUNG, S., A. LACOURLY & C. SARDA: Etude de l'évolution en chloroplastes des plantes étiolées d'orge I. Structure examinée par cryodécapage. *Z. Pflanzenphysiol.* 62, 1-16 (1970)
- PYLLOTIS, N. A. & D. J. GOODCHILD: The regreening of nitrogen-deficient *Chlorella fusca* II. Structural changes during synchronous regreening. *Arch. Microbiol.* 103, 259-270 (1975)
- ROBERTSON, D. & W. M. LAETSCH: Structure and function of developing barley plastids. *Plant Physiol.* 54, 148-159 (1974)
- SIMPSON, D. J., G. HØYER-HANSEN, N.-H. CHUA & D. VON WETTSTEIN: The use of gene mutants in barley to correlate thylakoid polypeptide composition with the structure of the photosynthetic membrane. *Proc. Fourth Internat. Cong. Photosynth.* Reading 537-548 (1977)
- SMILLIE, R. M., N. C. NIELSEN, K. W. HENNINGSEN & D. VON WETTSTEIN: Development of photochemical activity in chloroplast membranes. I. Studies with mutants of barley grown under a single environment. *Aust. J. Plant Physiol.* 4, 415-438 (1977)
- SPREY, B. & W. M. LAETSCH: Chloroplast envelopes of *Spinacia oleracea* L. III. Freeze-fracturing of chloroplast envelopes. *Z. Pflanzenphysiol.* 78, 360-371 (1976)
- STAEHELIN, L. A.: Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro. *J. Cell Biol.* 71, 136-158 (1976)
- WELLBURN, A. R., P. H. QUAIL & B. E. S. GUNNING: Examination of ribosome-like particles in isolated prolamellar bodies. *Planta* 134, 45-52 (1977)
- WELLBURN, A. R. & F. A. M. WELLBURN: Ul-

- trastructural morphogenesis in isolated etioplasts at high and low temperature and light flux. *Proc. Second Internat. Cong. Photosynth. Stresa Vol. III*, 2349-2365 (1971)
25. WETTSTEIN, D. VON: Developmental changes in chloroplasts and their genetic control. In: *Developmental Cytology* (Ed. D. Rudnick.) Ronald Press Co., New York, pp. 123-160 (1959)
26. WETTSTEIN, D. VON & A. KAHN: Macromolecular physiology of plastids. *Proc. Eur. Reg. Conf. Electron Microsc. Delft Vol. III*, 1051-1054 (1960)
27. WETTSTEIN, D. VON, K. W. HENNINGSSEN, J. E. BOYNTON, C. G. KANNANGARA & O. F. NIELSEN: The genic control of chloroplast development in barley. In: *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (Ed.: N. K. Boardman, A. W. Linnane & R. M. Smillie.) North-Holland, Amsterdam, pp. 205-223 (1971)