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Localization of Filipin-Sterol Complexes in the Membranes of *Beta vulgaris* Roots and *Spinacia oleracea* Chloroplasts[1]

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ABSTRACT

Filipin was used as a cytochemical probe for membrane sterols in the root storage tissue of the red beet *Beta vulgaris* L. and the chloroplasts of *Spinacia oleracea* L. In unfixed beet tissue, filipin lysed the cells. Freeze-fracture replicas revealed that the filipin-sterol complexes were tightly aggregated in the plasma membrane, while in thin section the complexes corrugated the plasma membrane. If the cells were fixed with glutaraldehyde prior to the filipin treatment, the cell structure was preserved. Filipin-induced lesions were dispersed or clustered loosely in the plasma membrane. A few filipin-sterol complexes were observed in the tonoplast. In spinach chloroplasts, filipin-sterol complexes were limited to the outer membrane of the envelope and were not found in the inner membrane of the envelope or in the lamellar membranes. If the filipin-sterol complexes accurately mapped the distribution of membrane sterols, then sterol was located predominantly in the plasma membrane of the red beet and in the outer membrane of the chloroplast envelope. Furthermore, the sterol may be heterogenously distributed laterally in both these membranes.

Sterols help to maintain membrane structure and function. It is important therefore to define which cellular membranes contain sterols and how the sterol is distributed within these membranes (see 7, 12 for reviews and references therein). Biochemical techniques have revealed that the sterols may be heterogeneously distributed between and within cellular membranes (12). Unfortunately, these methods are necessarily limited to those membranes which can be isolated in sufficient purity and quantity so that small differences in sterol composition are not obscured by contaminants. Cytochemical methods can often circumvent these limitations. Autoradiography with tritiated cholesterol (29) and the localization of cholesterol osmate by transmission electron microscope (16) have been tried, but the most effective and popular cytochemical probe for membrane sterol has been the use of filipin coupled with freeze-fracture electron microscopy. Filipin complexes with sterols (see 31 for review) to form characteristic lesions in the membrane which can be detected by freeze-fracture electron microscopy (10, 18, 47, 48).

Filipin has been used frequently to localize sterols in animal cell membranes (1, 2, 10, 11, 22–24, 30, 32–34, 39, 41–44). Although little is known about the function of sterols in plants (see 13, 14 for reviews) and plant membranes are often difficult to isolate for biochemical studies, filipin has rarely been used to map sterols in plant membranes (21, 40). In the present study, we used filipin to localize sterols in the membranes of red beet roots and spinach chloroplasts. The red beet was selected because the leakage of betacyanin, the red pigment confined to the beet vacuole, from the tissue could be used to monitor the penetration of filipin into the tissue (28). In addition, the storage tissue of red beet is composed mainly of large parenchyma cells with large central vacuoles and thin layers of cytoplasm sandwiched between the tonoplast and plasma membrane. The uniformity of the tissue simplified the ultrastructural work.

We also treated spinach chloroplasts with filipin to clarify the localization of sterols in chloroplast membranes. Sterol is present in chloroplasts in very small amounts with approximately two molecules sterol to 100 molecules of Chl while phosphatidylethanolamine, which may be a negative marker for chloroplasts (9), is present in approximately three molecules to 100 molecules of Chl (see 17 for review and references therein). The low analytical values for sterol content raised the possibility that the sterol present in chloroplast preparations was due to contamination. Poincelot (36) found sterol in a chloroplast envelope fraction and suggested that sterol was present only in the envelope and not in the lamellar membranes of the chloroplast. Only recently (6) has it been possible to separate the outer and inner membranes of the chloroplast envelope, but the sterol distribution between these membranes has not been determined.

MATERIALS AND METHODS

Red beets (*Beta vulgaris* L.) were purchased from a local market. Cores were cut from the roots with a cork borer (7 mm in diameter), and the cores were sectioned into discs about 1 mm in thickness (28). The discs were diced into cubes (approximately 1 mm^3) and washed for 1 h with Sørenson's phosphate buffer (20 mM, pH 7.2). This wash and all subsequent treatments for both the beet tissue and chloroplasts were done at room temperature. For the filipin experiments, half of the cubes were treated with 2 ml of a buffered filipin solution (40 μg filipin/ml, 1% [v/v] methanol), while the remaining cubes were placed in a buffered control solution (1% [v/v] methanol). The filipin was kindly provided by Dr. G. B. Whitfield, Jr., from the Upjohn Company, Kalamazoo, MI. The beet cubes were incubated in the filipin and control solutions for 0.5 to 2.5 h. After treatment, the filipin and control solutions were removed and buffer added. Cubes for freeze-fracture electron microscopy were quickly frozen and stored in liquid N_2 until used (25). For thin-section experiments, sample cubes were fixed in buffered 2.5% (v/v) glutaraldehyde for 1 h, post-fixed with buffered 1% (w/v) osmium tetroxide for 1 h, dehydrated in a graded series of acetone, and embedded in Spurr's resin (46). Thin sections were stained with aqueous uranyl acetate and Reynold's lead (38). In some of the experiments, the beet cubes were fixed prior to the filipin treatment with buffered 2.5% (v/v) glutaraldehyde for 1 h. For freeze-fracture experiments, the fixed cubes were also cryoprotected with buffered 20% (v/v) glycerol after the filipin treatment.

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Chloroplasts from *Spinacia oleracea* L. were isolated from either store-bought or hydroponically grown spinach (27). Intact chloroplasts were removed from Percoll (Pharmacia) step gradients (27) and mixed with enough resuspension buffer (0.33 M sorbitol; 0.5 mM Tris, pH 7.5) to give a Chl concentration of approximately 0.2 mg Chl/ml suspension. The Chl concentration was determined by the method of Whatley and Arnon (49). Aliquots (0.9 ml) of the chloroplast suspension were fixed with 0.1 ml of 25% (v/v) glutaraldehyde added directly to the aliquot. The chloroplasts were fixed for 0.5 h, recovered by centrifugation (1,900*g* for 5 min or 2,200*g* for 20 s), and resuspended in 2 ml of either a filipin solution (100 μg filipin/ml, 1% [v/v] dimethyl sulfoxide in resuspension buffer) or a control solution (1% [v/v] dimethyl sulfoxide in resuspension buffer). After 30 min, the chloroplasts were pelleted by centrifugation and portions of the pellet frozen for the freeze-fracture microscopy.

Both spinach and red beet samples were freeze-fractured with a Balzers freeze-etching machine (BAF 301) as described by Moor and Mühlethaler (25). Replicas were cleaned with chromic acid or by an alcoholic KOH technique (12% [w/v] KOH in 95% ethanol followed by sulfuric and chromic acid washes [35]) and picked up on Formvar-coated grids. Replicas and thin sections were examined using a Philips EM-400 microscope. Micrographs of replicas are presented with the shadow direction from the bottom to the top of the micrograph, and the nomenclature of Branton *et al.* (5) has been used to designate the membrane fracture faces.

RESULTS

Filipin promoted the loss of betacyanin from red beet cubes as shown by the increase in *A* at 530 nm (Fig. 1A). Concurrently, the absorbance of free filipin at 355 nm dropped as the filipin complexed with sterol (28). Little or no change in the absorbance at either wavelength was detected in the control. When the beet cubes were examined visually after the experiment, some of the larger cubes treated with filipin had clear margins and pink or red centers, while smaller cubes had completely lost their pigment. The control cubes were uniformly red. Evidently the filipin had not penetrated into the interior of the larger cubes. Thin sections of the cubes confirmed that not all of the cells may have been exposed to the filipin because the ultrastructural changes described below were confined to the margins of the cubes.

Large intact parenchyma cells were observed in the thin sections of the control cubes (Fig. 2A). Most of the cell volume was occupied by the large central vacuole which compressed the cytoplasm into a thin layer against the cell wall. The most prominent cellular membranes were the plasma membrane and tonoplast, and these were the membranes we studied. When treated with filipin, the cells lysed (Fig. 2B). The plasma membrane no longer pressed against the cell wall and contained numerous filipin-sterol complexes which gave it a characteristic 'corrugated' morphology when seen in thin section (Fig. 2B; Ref. 41). The tonoplast ruptured and was no longer identifiable, although the membranous vesicles within the lumen of the lysed cells may be derived from the tonoplast and perhaps other intracellular membranes. The loss of the tonoplast integrity may have helped lyse the cells. Mudd and Kleinschmidt (28) demonstrated that protein and nucleotides as well as betacyanin leaked from the beet tissue discs treated with filipin and suggested that membrane sterols may have been involved. Our results confirmed the action of filipin on membrane sterols and showed that the leakage of cellular constituents was coupled with cell lysis. We could not determine if the filipin-induced lesion which initiated the cell lysis was in the plasma membrane or in the tonoplast.

Freeze-fracture studies corroborated the thin section work. In control tissue, the plasma membrane and tonoplast were seen as smooth sheets of membrane close to the cell wall (Fig. 3A). When the tissue was treated with filipin, the cell compartmentation was lost. The plasma membrane pulled away from the cell wall (Fig. 3B) and contained numerous filipin-sterol complexes (Fig. 3, B and C). The filipin-induced lesions were similar in size and shape to those described previously in the literature (10). The filipin-sterol complexes were most frequently aggregated into tight clusters (Fig. 3, B and C). In some cases, membranes were found completely covered with complexes or infrequently with just a few small clusters of complexes. The tonoplast ruptured and could not be identified. Vesicles of unknown origin, some of which contained fillipin-sterol complexes, were found in the cell lumen.

Red beet cubes were also fixed prior to filipin treatment to preserve the cell ultrastructure and to block the possible artefactual aggregation of filipin-sterol complexes (41). Fixation with glutaraldehyde disrupted the permeability properties of the red beet membranes and allowed betacyanin to leak out of both filipin-treated and untreated cubes (Fig. 1B). However, the filipin still complexed with sterol (drop in *A* at 355 nm) and accelerated the efflux of betacyanin (increase in *A* at 530 nm). Visual inspection of the cubes revealed that the cubes treated with filipin had larger margins cleared of the red pigment than did the control cubes.

Fixation of the cells prior to filipin treatment preserved the cell ultrastructure and few differences were found between the control (Fig. 2C) and filipin-treated (Fig. 2D) samples in thin section. The dramatic corrugated plasma membrane seen in unfixed tissue (Fig. 2B) was not observed, although there were some indications of filipin-sterol complexes in the plasma membrane (circled area in Fig. 2D) and possibly the tonoplast of filipin-treated cells. In freeze-fracture, filipin-sterol complexes formed pits in the exoplasmic fracture face and bumps in the protoplasmic fracture face of the plasma membrane (Fig. 4B). The tightly aggregated clusters of filipin-sterol complexes observed in unfixed tissue (Fig. 3, B and C) were not formed in the fixed tissue. Instead, the complexes were dispersed (Fig. 4A) or aggregated into loose clusters (Fig. 4B). Some fractures of the plasma membrane contained more filipin-sterol complexes than others (compare Fig. 4B and 4D). Membrane fracture faces which could be unambiguously identified as tonoplast were uncommon, but when detected, they contained filipin-sterol complexes although at a much lower density than the plasma membrane (Fig. 4D).

Intact chloroplasts treated with filipin contained filipin-sterol complexes only in the outer membrane of the chloroplast envelope (Fig. 5). In some but not all experiments with filipin, the outer membrane of the envelope detached from the chloroplast and could be identified easily in both cross fracture and face view (Fig. 5A). The detachment of the outer membrane along with the fact that the outer membrane contained far fewer intramembranous particles than either the inner membrane of the envelope or the thylakoid membrane system (45) allowed the outer membrane to be identified unambiguously by freeze-fracture. Clusters of filipin-sterol complexes (in one experiment, the complexes were dispersed) were found in the outer membrane (Fig. 5). Filipin-sterol complexes were never detected in the inner membrane of the envelope or in the thylakoid system. The lack of complexes in these membranes was not due to a problem of penetration of the filipin into the chloroplast. The outer membrane of the chloroplast envelope is freely permeable to molecules smaller than 10,000 D (see 8, 15 for reviews) and the outer membrane in some of the experiments may have been disrupted (Fig. 5A). Filipin should have been able to penetrate into the intermembrane space and interact with the inner membrane of the envelope. Broken chloroplasts, which contained only the thylakoid membrane system, were recovered from the Percoll gradient and treated with filipin, but no filipin-sterol complexes were found in these membranes. Unfixed chloroplasts contained clustered filipin-sterol complexes only in the outer membrane of the envelope. The temperature at which the chloroplasts were fixed and treated did not have a consistent effect on the distribution of the intramembrane particles

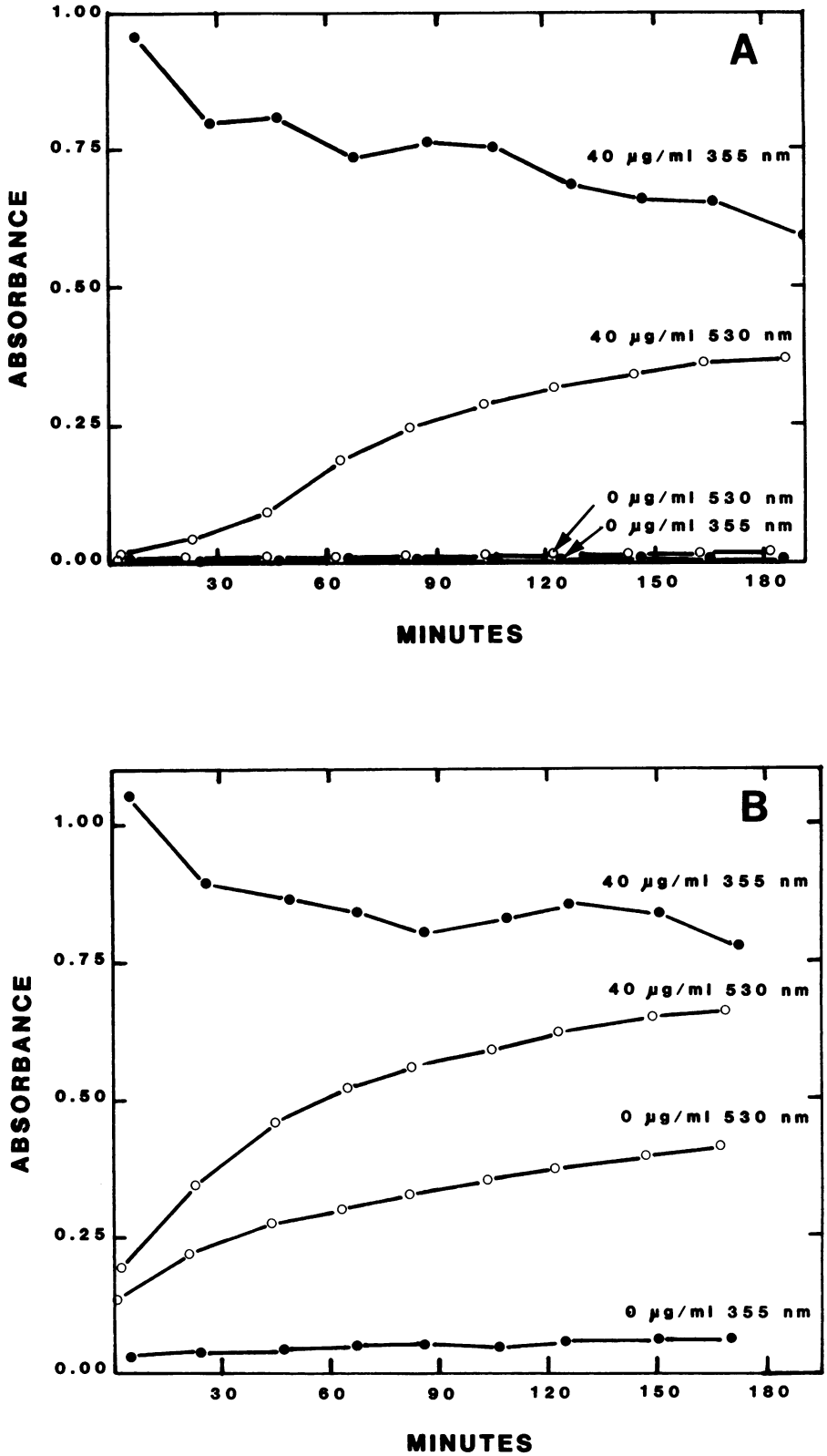

FIG. 1. Effects of filipin on the leakage of betacyanin from fixed and unfixed red beet cubes. A, Cubes from red beets (approximately 2/3 of 4 discs) were prepared as described in "Materials and Methods." Both filipin-treated (40 µg/ml) and untreated (0 µg/ml) cubes were incubated in a water bath at 26°C on a shaker. Periodic readings were taken at 355 and 530 nm to measure filipin-sterol complex formation and betacyanin leakage respectively. B, In a separate experiment, cubes were treated as in A, except that the cubes were fixed for 0.5 h with buffered 2.5% (v/v) glutaraldehyde and then rinsed with buffer for 15 min prior to treatment with the filipin and control solutions.

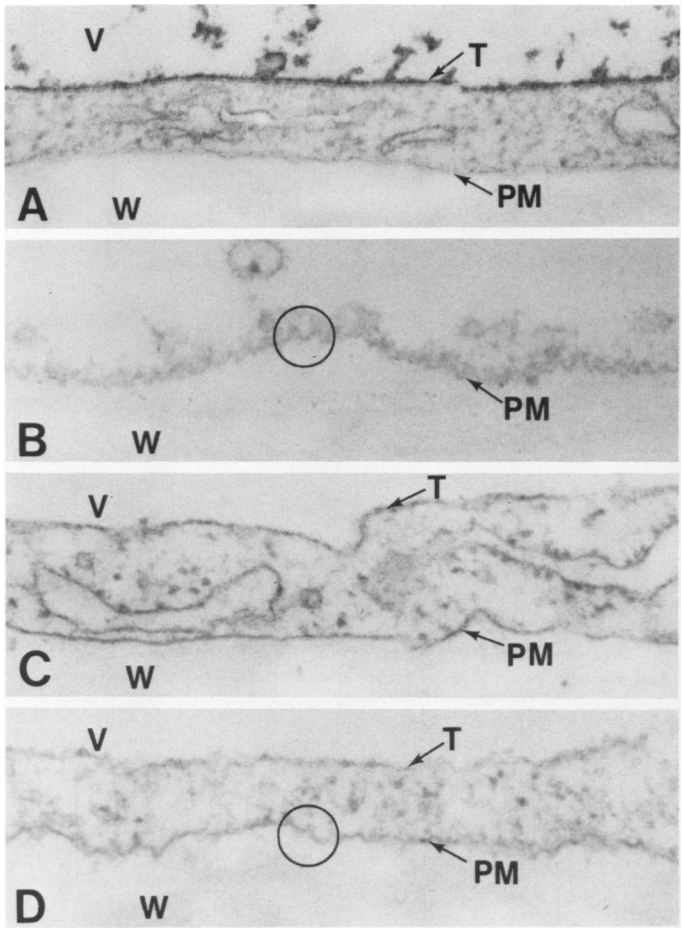

FIG. 2. Thin sections of filipin-treated and untreated red beet cubes. A, Red beet tissue incubated in buffered 1% (v/v) methanol prior to fixation for thin sections. B, Beet cubes treated with 40 μg/ml filipin in buffered 1% methanol prior to fixation. Circled area shows region 'corrugated' with filipin-sterol complexes. C, Red beet tissue fixed with glutaraldehyde prior to incubation in buffered 1% methanol. D, Red beet cube fixed with glutaraldehyde and then treated with 40 μg/ml filipin in buffered 1% methanol. Circled area in D shows possible filipin-sterol complex. A–D, × 70,000; W, cell wall; PM, plasma membrane; T, tonoplast; V, vacuole.

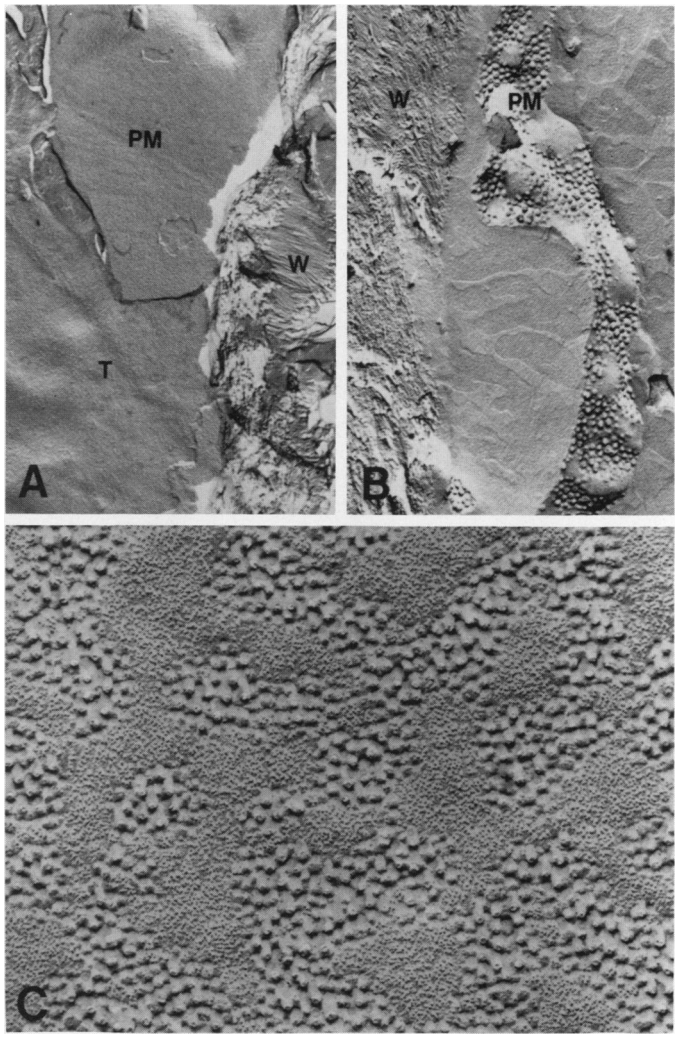

FIG. 3. Freeze-fracture replicas of unfixed red beet cubes. A, Control tissue incubated in buffered 1% methanol. Protoplasmic fracture face of the plasma membrane (PM) with the underlying exoplasmic fracture face of the tonoplast (T) is shown along with overlying wall material (W). B, Exoplasmic fracture face of the plasma membrane (PM). Note the clustered filipin-sterol complexes and separation of the PM from the cell wall (W). C, Enlargement of protoplasmic fracture face of the plasma membrane showing aggregation of filipin-sterol clusters. A and B, × 26,000; C, × 70,000.

or filipin-sterol complexes (Fig. 5, A and B fixed and treated at room temperature; Fig. 5C, at 0°C).

DISCUSSION

At present, it is often difficult if not impossible to isolate some plant cell membranes in sufficient quantity or purity to perform reliable assays for membrane sterol. Filipin provides a cytochemical approach to determine which cellular membranes contain sterol. The bulk of the membrane sterol in animal cells is located in the plasma membrane (see 12 for review and references therein) and this appears also to be true of plant cells (see 20 for review and references therein). Filipin-sterol complexes were most numerous in the plasma membrane of red beet cells and much scarcer in the tonoplast of the same cells. The distribution of the complexes agreed with the expected distribution of sterol between these two membranes and confirmed the presence of sterol in the tonoplast detected by biochemical methods (19). Some of the beet plasma membranes contained greater numbers of the filipin-sterol complexes than others, and this may reflect differences in the sterol content of plasma membranes of cells of the same or different tissue type. A similar heterogenity exists in animals because apical plasma membranes from adjacent cells of the toad bladder contained different numbers of filipin-sterol complexes (33). This type of sterol heterogeneity is missed by biochemical techniques.

We found filipin-sterol complexes only in the outer membrane of the chloroplast envelope and not in the inner membrane of the envelope or in the lamellar membranes. These results confirmed that the small amounts of sterol found in chloroplast preparations (17) were not due only to contaminants and that the sterol present was in the envelope (36). The absence of filipin-sterol complexes from the inner membrane of the chloroplast envelope and the thylakoids does not exclude the possibility that small amounts of sterol may be present in these membranes. Studies of liposomes prepared with different cholesterol:phosphatidylcholine ratios showed that filipin-sterol complexes were detected by freeze-fracture at a mole ratio of 1:20, sometimes at a ratio of 1:50, but not at a ratio of 1:100 (10). From Poincelot's data (36) for the chloroplast envelope, the ratio of sterol:polar lipid in the envelope can be calculated to be about 1:24. This ratio would be even

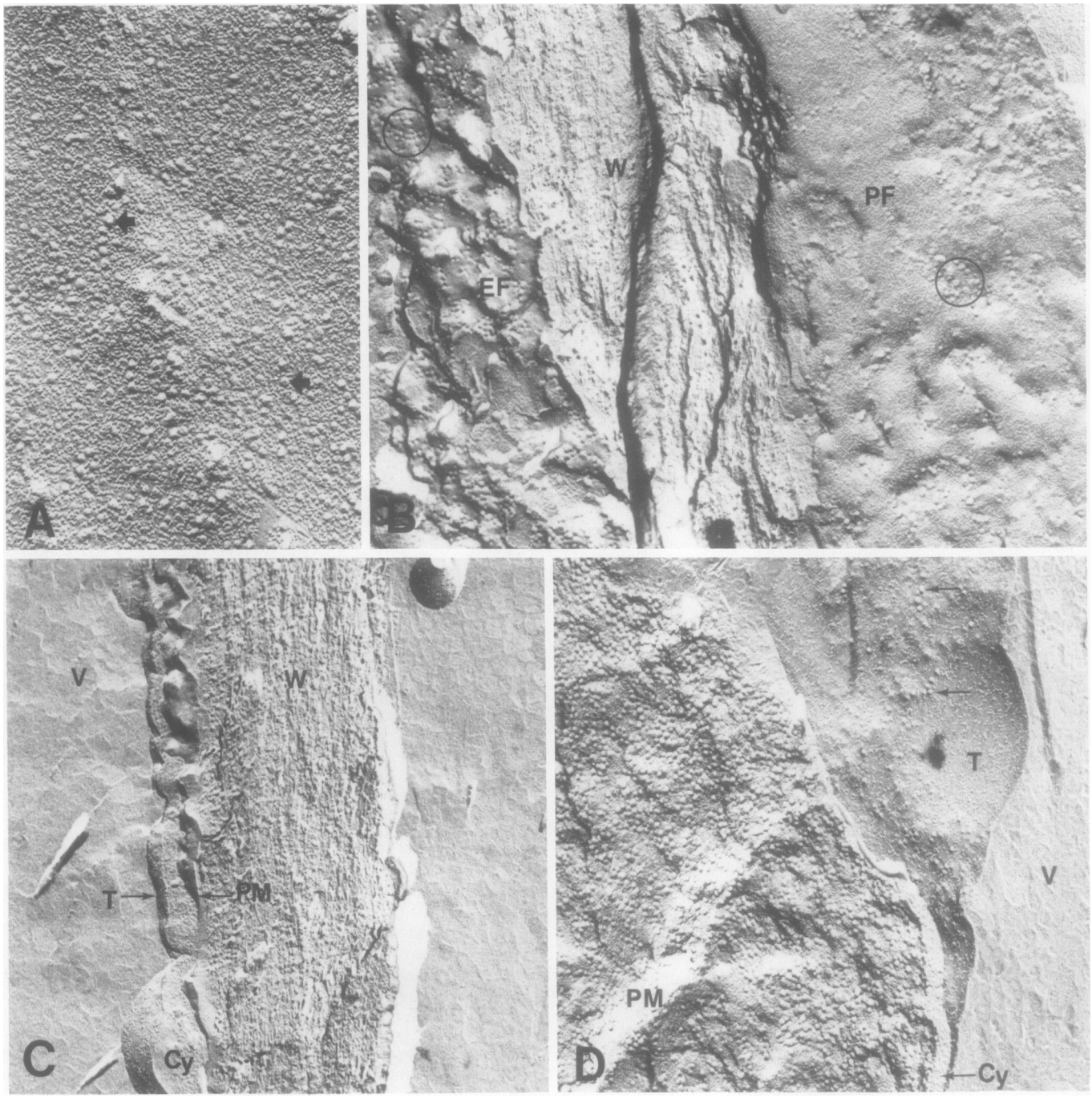

FIG. 4. Freeze-fracture replicas of fixed and cryoprotected red beet tissue. A, Portion of protoplasmic fracture face of plasma membrane with dispersed filipin-sterol complexes (arrows). B, Exoplasmic (EF) and protoplasmic (PF) fracture faces of the plasma membranes of two adjacent cells along with the intervening wall (W). The filipin-sterol complexes are loosely clustered (circled areas) and appear mainly as pits in the EF face and as bumps in the PF face. C, Cross-fracture of control cell showing close juxtaposition of wall (W), exoplasmic fracture face of plasma membrane (PM), cytoplasm (Cy), protoplasmic fracture face of the tonoplast (T), and vacuole (V). D, This fracture presents face views of the membranes shown in cross-fracture in D and has been treated with filipin to localize sterols. The protoplasmic fracture face of the plasma membrane (PM) is shown with the underlying exoplasmic fracture face of the tonoplast (T). Sandwiched between these membrane leaflets is the cytoplasm (Cy). Filipin-sterol complexes are denoted by arrows and are more abundant in the plasma membrane than in the tonoplast. A, × 42,500; B–D, × 26,000.

greater if the sterol was found only in the outer membrane. Filipin detected the sterol in the outer membrane of the envelope but may have missed small amounts present in the other chloroplast membranes. While our work on the spinach chloroplast was in progress, Melkonian *et al.* (21) reported that they found filipin-sterol complexes in the chloroplast envelopes of *Euglena* and *Ochromonas* but not in the envelopes of isolated pea and spinach chloroplasts. We do not know the reason for the discrepancy between their results and ours for the spinach chloroplast.

The role of the chloroplast sterol is not understood. Filipin at a high concentration will inhibit the photochemical activity (3, 4), but this inhibition may be the result of a detergent action of the filipin rather than a specific interaction of filipin with sterol (26). Our results showed that sterol is probably confined to the outer membrane of the chloroplast envelope, and lower concentrations of the filipin may be needed to define the function of the envelope

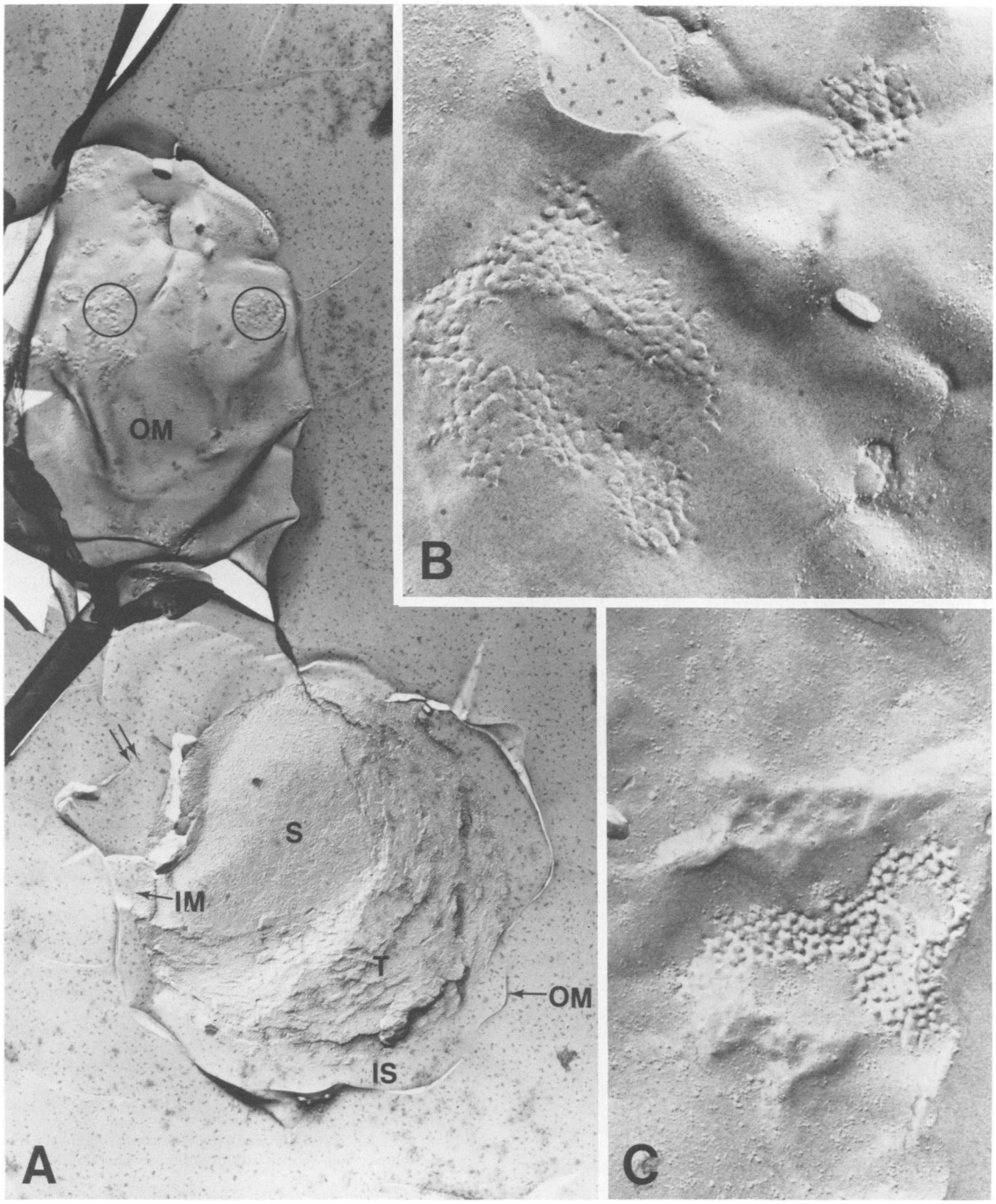

FIG. 5. Freeze-fracture replicas of spinach chloroplasts exposed to filipin. A, The chloroplast in the lower portion of the micrograph has been cross-fractured and reveals the stroma (S) and the thylakoid (T) system of the chloroplast as well as the chloroplast envelope which is composed of an inner (IM) and outer (OM) membrane with the enclosed intermembrane space (IS). The outer membrane (OM) detached from the chloroplast and may have broken (arrows). The chloroplast in the upper portion of the micrograph contains filipin-sterol complexes (circled areas) in the exoplasmic fracture face of the outer membrane (OM) of the chloroplast envelope. B, Exoplasmic fracture face of the outer membrane of the chloroplast envelope which shows the filipin-sterol complexes as a cluster of pits. The intramembranous particles are also aggregated. C, Protoplasmic fracture face of the outer membrane of the chloroplast envelope. Once again the filipin-sterol complexes are clustered, but in this fracture face they appear as clusters of protuberances. Filipin-sterol complexes also formed pits in the protoplasmic fracture face and bumps in the exoplasmic fracture face of the outer membrane of the chloroplast envelope. A, × 15,000; B and C, × 42,500.

sterol in the chloroplast. The presence of sterol only in the outer membrane of the chloroplast and the large amounts of phosphatidylcholine present in this membrane (6) are consistent with the endosymbiont model for the origin of the chloroplast. Both these lipids are much more prevalent in eukaryotes than in prokaryotes and would be expected to be more abundant in those membranes which purportedly are derived from the ancestral eukaryote (21, 50). We can not explain the distribution of the sterol in the chloroplast because presumably cellular sterol can come into contact with all the chloroplast membranes but is found only in the outer membrane of the envelope.

Filipin can be used to probe the lateral distribution of sterols in

membranes, and it has shown that the sterol may not be scattered homogenously in the plane of the animal plasma membrane. Filipin-sterol complexes may be excluded from certain regions (coated pits [11, 23, 24]; particle aggregates induced by vasopressin [33]; neuromuscular junctions [30]; desmosomes, gap junctions, and tight junctions [10, 43]; areas of smooth muscle [22]; plaques in the plasma membrane of the rat urothelium—however, the plaques may contain sterol since they react with digitonin [44]), clustered in other areas (rod disc membranes [2]; areas of the plasma membrane overlying secretory granules [33]), or homogenously distributed in the plasma membrane (41). In addition, lipid phase separations changed the distribution of filipin-sterol complexes (42). Intracellular organelles such as the Golgi apparatus (34) and nucleus (1, 39) also contained areas enriched in filipin-sterol complexes. Filipin-sterol complexes may also be aggregated in the plasma membrane of red beets and the chloroplast envelope (see "Results"). The presence of sterol-enriched and depleted regions in the plasma membrane of single cells coupled with the possibility of varied sterol compositions between the plasma membrane of the same cell type could interfere with the 'digitonin shift' experiments proposed by Quail (37) to separate and identify the plasma membrane from plants.

Whether filipin-sterol complexes form pits or bumps in the membrane may indicate the transbilayer distribution of the membrane sterol (32). Both the plasma membrane of the beet tissue and the outer membrane of the chloroplast envelope often contained predominant bumps in one fracture face and pits in the other fracture face, and this may reflect an asymmetrical distribution of sterol between the two leaflets of the bilayer (Figs. 4B; 5, B and C). However, much more work is needed with membranes of known sterol asymmetry before a definitive correlation between transbilayer asymmetry and the morphology of the filipin-sterol complex can be made.

Filipin has proved useful as a cytochemical probe for membrane sterols. Hopefully, the combined use of filipin as a cytochemical and biochemical probe for sterol will better define the role of sterols in plant membranes.

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LITERATURE CITED

1. ALROY J, FB MERK, V GOYAL, A UCCI 1981 Heterogeneous distribution of filipin-sterol complexes in nuclear membranes. Biochim Biophys Acta 649: 239–243
2. ANDREWS LD, AI COHEN 1979 Freeze-fracture evidence for the presence of cholesterol in particle-free patches of basal disks and the plasma membrane of retinal rod outer segments of mice and frogs. J Cell Biol 81: 215–228
3. BISHOP DG 1973 Inhibition of photochemical activity in chloroplasts by the polyene antibiotic, filipin. Arch Biochem Biophys 154: 520–526
4. BISHOP DG 1974 The effect of polyene antibiotics on photosynthetic electron transfer. J Exp Bot 25: 491–502
5. BRANTON D, S BULLIVANT, NB GILULA, MJ KARNOVSKY, H MOOR, K MÜHLETHALER, DH NORTHCOTE, L PACKER, B SATIR, P SATIR, V SPETH, LA STAEHELIN, RL STEERE, RS WEINSTEIN 1975 Freeze-etching nomenclature. Science 190: 54–56
6. CLINE K, J ANDREWS, B MERSEY, EH NEWCOMB, K KEEGSTRA 1981 Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc Natl Acad Sci USA 78: 3595–3599
7. DEMEL RA, B DE KRUYFF 1976 The function of sterols in membranes. Biochim Biophys Acta 457: 109–132
8. DOUCE R, J JOYARD 1979 Structure and function of the plastid envelope. Adv Bot Res 7: 1–116
9. DUBACQ J-P, J-C KADER 1978 Free flow electrophoresis of chloroplasts. Plant Physiol 61: 465–468
10. ELIAS PM, DS FRIEND, J GOERKE 1979 Membrane sterol heterogeneity freeze-fracture detection with saponins and filipin. J Histochem Cytochem 27: 1247–1260
11. FUJITA H, K ISHIMURA, H MATSUDA 1981 Freeze-fracture images on filipin-sterol complexes in the thyroid follicle epithelial cell of mice with special regard to absence of cholesterol at the site of micropinocytosis. Histochemistry 73: 57–63
12. GREEN C 1977 Sterols in cell membranes and model membrane systems. *In* TW Goodwin, ed, International Review of Biochemistry, Biochemistry of Lipids II, Vol 14. University Park Press, Baltimore, pp 101–152
13. GRUNWALD C 1975 Plant sterols. Annu Rev Plant Physiol 26: 209–236
14. GRUNWALD C 1978 Function of sterols. Philos Trans R Soc Lond B 284: 541–558
15. HEBER U, HW HELDT 1981 The chloroplast envelope: structure, function, and role in leaf metabolism. Annu Rev Plant Physiol 32: 139–168
16. HIGGINS JA, NT FLORENDO, RJ BARRNETT 1973 Localization of cholesterol in membranes of erythrocyte ghosts. J Ultrastruct Res 42: 66–81
17. KIRK JTO, RAE TINLEY-BASSET 1978 Chloroplast lipids. *In* The Plastids: Their Chemistry, Structure, Growth and Inheritance. Elsevier/North-Holland Biomedical Press, New York, pp 50–63
18. KITAJIMA Y, T SEKIYA, Y NOZAWA 1976 Freeze-fracture ultrastructural alterations induced by filipin, pimaricin, nystatin, and amphotericin B in the plasma membranes of *Epidermophyton*, *Saccharomyces* and red blood cells. A proposal of models for polyene-ergosterol complex-induced membrane lesions. Biochim Biophys Acta 445: 452–465
19. MARTY F, D BRANTON 1980 Analytical characterization of beetroot vacuole membrane. J Cell Biol 87: 72–83
20. MAZLIAK P 1977 Glyco and phospholipids of biomembranes in higher plants. *In* M Tevini, HK Lichtenthaler, eds, Lipids and Lipid Polymers in Higher Plants. Springer-Verlag, New York, pp 48–74
21. MELKONIAN M, H ROBENEK, M STEUP 1981 Occurrence and distribution of filipin-sterol complexes in chloroplast envelope membranes of algae and higher plants as visualized by freeze-fracture. Protoplasma 109: 349–358
22. MONTESANO R 1979 Inhomogeneous distribution of filipin-sterol complexes in smooth muscle cell plasma membrane. Nature 280: 328–329
23. MONTESANO R, A PERRELET, P VASSALLI, L ORCI 1979 Absence of filipin-sterol complexes from large coated pits on the surface of cultured cells. Proc Natl Acad Sci USA 76: 6391–6395
24. MONTESANO R, P VASSALLI, L ORCI 1981 Structural heterogeneity of endocytic membranes in macrophages as revealed by the cholesterol probe, filipin. J Cell Sci 51: 95–107
25. MOOR H, K MÜHLETHALER 1963 Fine structure in frozen-etched yeast cells. J Cell Biol 17: 609–628
26. MUDD JB 1980 Sterol interconversions. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 4. Academic Press, New York, pp 509–534
27. MUDD JB, R DEZACKS 1981 Synthesis of phosphatidylglycerol by chloroplasts from leaves of *Spinacia oleracea* L. (spinach). Arch Biochem Biophys 209: 584–591
28. MUDD JB, MG KLEINSCHMIDT 1970 Effect of filipin on the permeability of red beet and potato tuber discs. Plant Physiol 45: 517–518
29. MURPHY JR 1965 Erythrocyte metabolism. VI. Cell shape and the location of cholesterol in the erythrocyte membrane. J Lab Clin Med 65: 756–774
30. NAKAJIMA Y, PC BRIDGMAN 1981 Absence of filipin-sterol complexes from the membranes of active zones and acetylcholine receptor aggregates at frog neuromuscular junctions. J Cell Biol 88: 453–458
31. NORMAN AW, AM SPIELVOGEL, RG WONG 1976 Polyene antibiotic-sterol interaction. Adv Lipid Res 14: 127–170
32. ORCI L, RG MILLER, R MONTESANO, A PERRELET, M AMHERDT, P VASSALLI 1980 Opposite polarity of filipin-induced deformations in the membrane of condensing vacuoles and zymogen granules. Science 210: 1019–1021
33. ORCI L, R MONTESANO, D BROWN 1980 Heterogeneity of toad bladder granular cell luminal membranes distribution of filipin-sterol complexes in freeze-fracture. Biochim Biophys Acta 601: 443–452
34. ORCI L, R MONTESANO, P MEDA, F MALAISSE-LAGAE, D BROWN, A PERRELET, P VASSALLI 1981 Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. Proc Natl Acad Sci USA 78: 293–297
35. PLATT-ALOIA KA, WW THOMSON 1982 Freeze-fracture of intact plant tissues. Stain Technol. In press
36. POINCELOT RP 1976 Lipid and fatty acid composition of chloroplast envelope membranes from species with differing net photosynthesis. Plant Physiol 58: 595–598
37. QUAIL PH 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
38. REYNOLDS ES 1963 The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212
39. ROBENEK H, H GREVEN 1981 Freeze-fracture evidence for high cholesterol content in the nuclear membranes of a larval urodelan epidermis. Eur J Cell Biol 25: 131–135
40. ROBENEK H, M MELKONIAN 1981 Sterol-deficient domains correlate with intramembrane particle arrays in the plasma membrane of *Chlamydomonas reinhardii*. Eur J Cell Biol 25: 258–264
41. ROBINSON JM, MJ KARNOVSKY 1980 Evaluation of the polyene antibiotic filipin as a cytochemical probe for membrane cholesterol. J Histochem Cytochem 28: 161–168
42. SEKIYA T, KITAJIMA Y, Y NOZAWA 1979 Effects of lipid-phase separation on the filipin action on membranes of ergosterol-replaced *Tetrahymena* cells, as studied by freeze-fracture electron microscopy. Biochim Biophys Acta 550: 269–278
43. SEVERS NJ 1981 Plasma membrane cholesterol in myocardial muscle and capillary endothelial cells. Distribution of filipin-induced deformations in freeze-fracture. Eur J Cell Biol 25: 289–299
44. SEVERS NJ, RC WARREN, SH BARNES 1981 Analysis of membrane structure in the transitional epithelium of rat urinary bladder. 3. Localization of cholesterol

using filipin and digitonin. J Ultrastruct Res 77: 160–188
45. SPREY B, WM LAETSCH 1976 Chloroplast envelopes of *Spinacia oleracea* L. III. Freeze-fracturing of chloroplast envelopes. Z Pflanzenphysiol 78: 360–371
46. SPURR AR 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43
47. TILLACK TW, SC KINSKY 1973 A freeze-etch study of the effects of filipin on liposomes and human erythrocyte membranes. Biochim Biophys Acta 323: 43–54
48. VERKLEIJ AJ, B DE KRUIJFF, WF GERRITSEN, RA DEMEL, LLM VAN DEENEN, PHJ VERVERGAERT 1973 Freeze-etch electron microscopy of erythrocytes, *Acholeplasma laidlawii* cells and liposomal membranes after the action of filipin and amphotericin B. Biochim Biophys Acta 291: 577–581
49. WHATLEY FR, DI ARNON 1963 Photosynthetic phosphorylation in plants. Methods Enzymol 6: 308–313
50. WHATLEY JM 1981 Chloroplast evolution—ancient and modern. Ann N Y Acad Sci 361: 154–165