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Plasmatubules: fact or artefact?

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Abstract. Plasmatubules are tubular evaginations of the plasmalemma associated with sites where high solute flux occurs between apoplast and symplast. Plasmatubules of the scutellar epithelial cells of germinating barley (*Hordeum vulgare* L.) have been examined following a variety of fixation methods. Of the aqueous fixations, primary aldehyde fixation with osmium post-fixation and osmium as the primary fixative gave comparable images, whilst potassium permanganate resulted in some distortion of the tissue in general including dilation of the tubular evaginations of the plasmalemma. Freeze-fixation and substitution with acetone and acetone-osmium gave images of the plasmalemma comparable to those obtained by the aqueous aldehyde and osmium methods. The similarity of structure with aldehyde or osmium and freezing as the primary fixation is taken to indicate that plasmatubules are real and not artefacts resulting from the fixation procedure.

Key words: *Hordeum* (scutellum, plasmatubule) – Plasmatubule – Scutellum (plasmatubule).

Introduction

Plasmatubules are tubular evaginations of the plasmalemma and have been reported in the scutellar epithelial cells of germinating barley (Harris et al. 1982) and phloem-associated parenchyma cells of mung-bean cotyledons (Harris 1981). It has been postulated that these amplifications of the plasmalemma surface area are specific to sites where there is high solute flux from the apoplast to the symplast for short periods of time (Harris 1981). Since the discovery of plasmatubules, however, some reservations have been raised concerning their reality – are they genuine cell features or artefacts of specimen fixation? Such problems have been encountered with previous work on other paramural bodies (e.g. Marchant and Robards 1968; Heath and Greenwood 1970; O'Brien 1972; Marchant and Moore 1973), In an attempt to resolve the controversy we have investigated the morphology and ultrastructure of plasmatubules in scutellar epithelial cells of germinating barley using a variety of aqueous fixation techniques and also freeze-substitution. The results presented illustrate essentially similar structures irrespective of the method of preparation indicating that plasmatubule structure is indeed real rather than a consequence of the fixation method.

Material and methods

Plant material.

Caryopses of *Hordeum vulgare* L. (cv. Maris Otter, Winter) were germinated for 3 d on 1.2% (w/v) water-agar at 22° C with 10 h illumination: 14 h dark cycles.

Transmission electron microscopy.

Aqueous fixation. Embryos were excised in 0.05 M sodium cacodylate buffer pH 7.0 and transverse scutellar sections taken and fixed following one of three protocols.

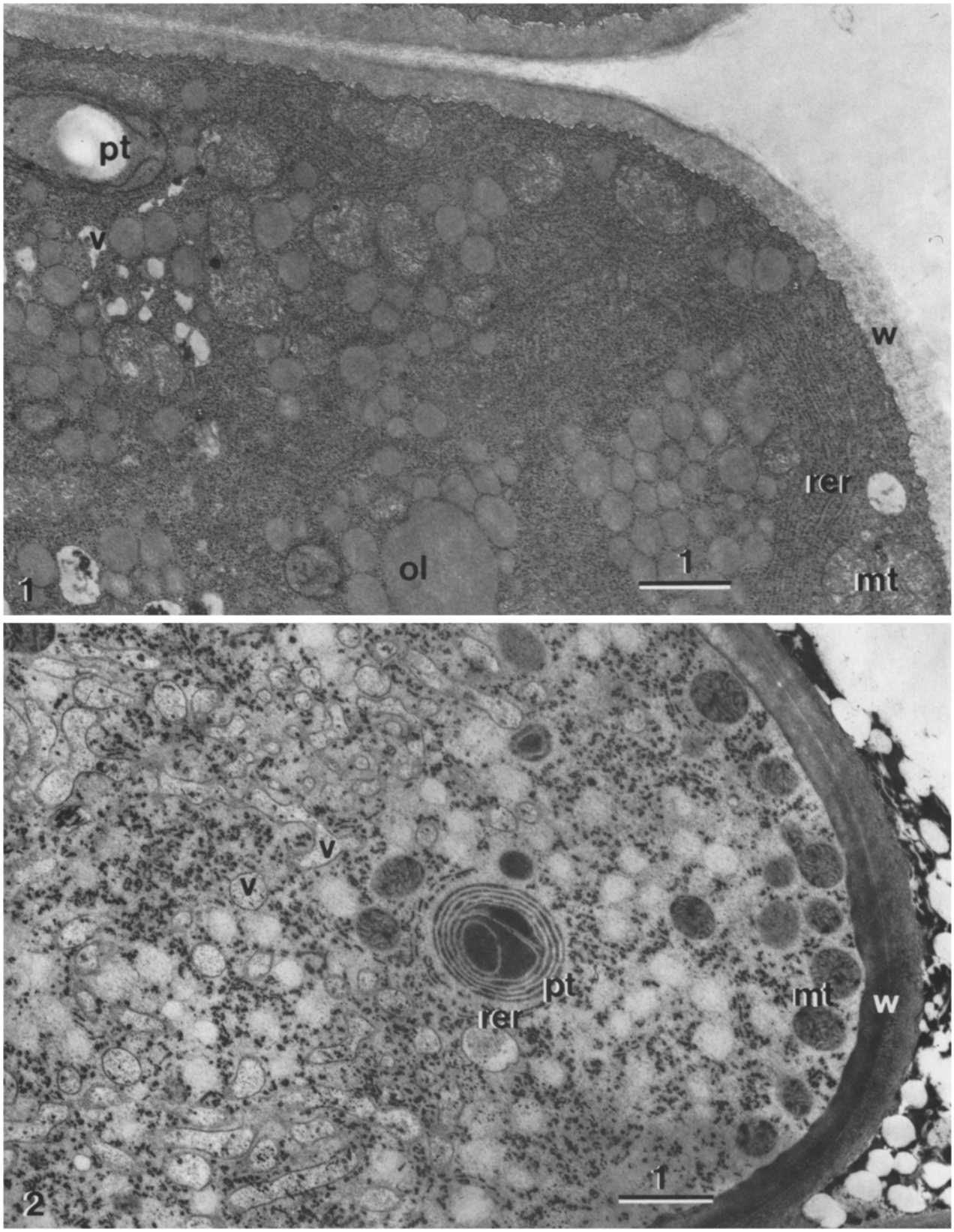
a) Conventional aldehyde fixation: tissue was fixed for 2 h in 2.5% glutaraldehyde, 1.5% paraformaldehyde in cacodylate buffer. Following a distilled water wash (2 × 15 min) the tissue was post-fixed for 1.5–2 h in 1% aqueous osmium tetroxide, dehydrated through an ethanolic series and embedded in Spurr resin.

b) Osmium fixation: tissue was fixed for 4–5 h in 1% aqueous osmium tetroxide, dehydrated through an ethanolic series and embedded in Spurr resin.

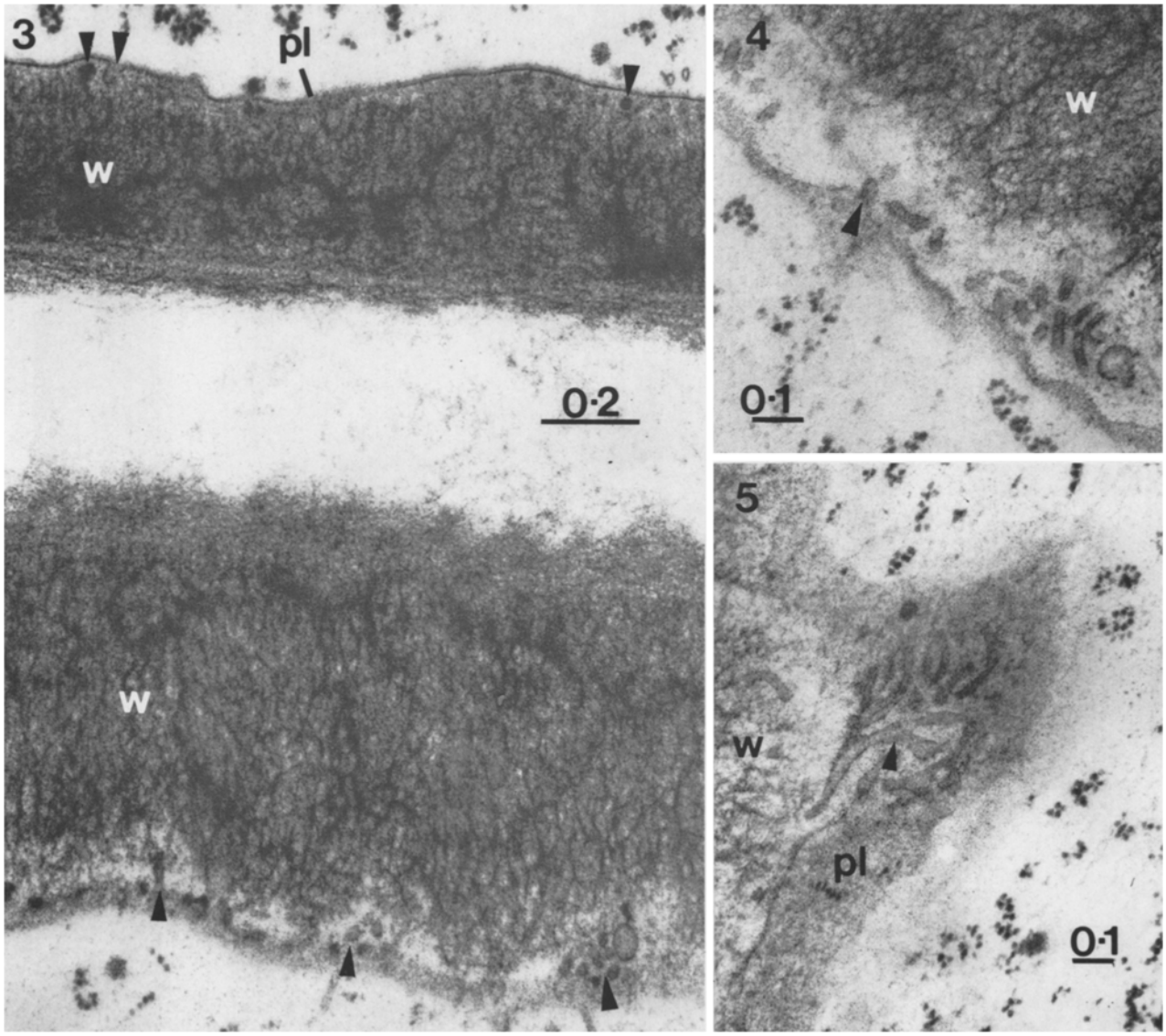
c) Permanganate fixation: tissue was fixed for 4–5 h in 2% aqueous potassium permanganate, dehydrated through an ethanolic series and embedded in Spurr resin.

All fixations, dehydrations and infiltrations were performed at room temperature.

Freeze-substitution. Scutella were sliced transversely and transferred to a vial of Freon 12 kept slushy above a dewar of liquid nitrogen. After approx. 10 min, 10–12 tissue slices were transferred, using cooled forceps, to a vial of slushy anhydrous acetone. The vial was capped and transferred to a box at −80° C. After 14 d, the acetone solution was replaced with an anhydrous solution of 2% osmium tetroxide (w/v) in acetone. The tissue was brought gradually, over a period of 15 h, from −80° C to −20° C. The tissue was then transferred to anhydrous acetone for 1 h at −20° C. The tissue was infiltrated



Figs. 1, 2. Longitudinal sections through scutellar epithelial cells of *Hordeum vulgare* imbibed for 3 d. Tissue post-stained with uranyl acetate and lead citrate. *mt*, Mitochondrion; *ol*, oleosome; *pt*, plastid; *rer*, rough endoplasmic reticulum; *v*, vacuole; *w*, wall. Scale bars in μm. **Fig. 1.** Epithelial cells after aldehyde fixation with osmium post-fixation. ×16000. **Fig. 2.** Epithelial cells after freeze-substitution and osmium post-fixation. ×16700



Figs. 3–5. Longitudinal sections through epithelial cells of *Hordeum vulgare* imbibed for 3 d. Tissue post-stained with uranyl acetate and lead citrate. Scale bars in μm. **Fig. 3.** Freeze-substituted material showing parts of two adjacent cells where the walls (*w*) have separated along the middle lamella. Plasmatubules (*darts*) are present in both cells but are more apparent in the lower cell where the wall-plasmalemma (*pl*) interface has been cut in glancing section. ×69300. **Fig. 4.** A glancing section through wall (*w*)-plasmalemma interface in freeze-substituted tissue showing continuity of plasmatubule with the plasmalemma (*dart*). ×75000. **Fig. 5.** Branching of plasmatubules (*dart*) in freeze-substituted tissue. ×58000

with 1:1 Spurr resin:anhydrous acetone, precooled to −20° C, with a gradual rise in temperature to 0° C over a 14-h period. The issue was subsequently infiltrated with Spurr resin for approx. 48 h at 4° C and then embedded.

Ultrathin sections (approx. 80 nm) were collected on formvar-coated grids, sequentially stained with saturated aqueous uranyl acetate (30 min) and alkaline lead citrate (15 min) before examination at 60 or 80 kV in an EM 400 electron microscope (Philips, Eindhoven, The Netherlands).

Results

Conventional aldehyde fixation. The structure of plasmatubules of barley scutellar epithelium following conventional aldehyde fixation with osmium tetroxide post-fixation has been described previously (Harris et al. 1982) and will only be summarised here. After 3 d imbition the scutellar epithelial cells have begun to separate along the middle lamellae which join their radial (anticlinal) walls. The cells contain numerous mitochondria, characteristically located peripherally, numerous vacuoles and oleosomes, plastids and rough cisternal endoplasmic reticulum (e.g. Fig. 1). The plasmalemma has an irregular profile and the periplasmic space contains numerous plasmatubules in a

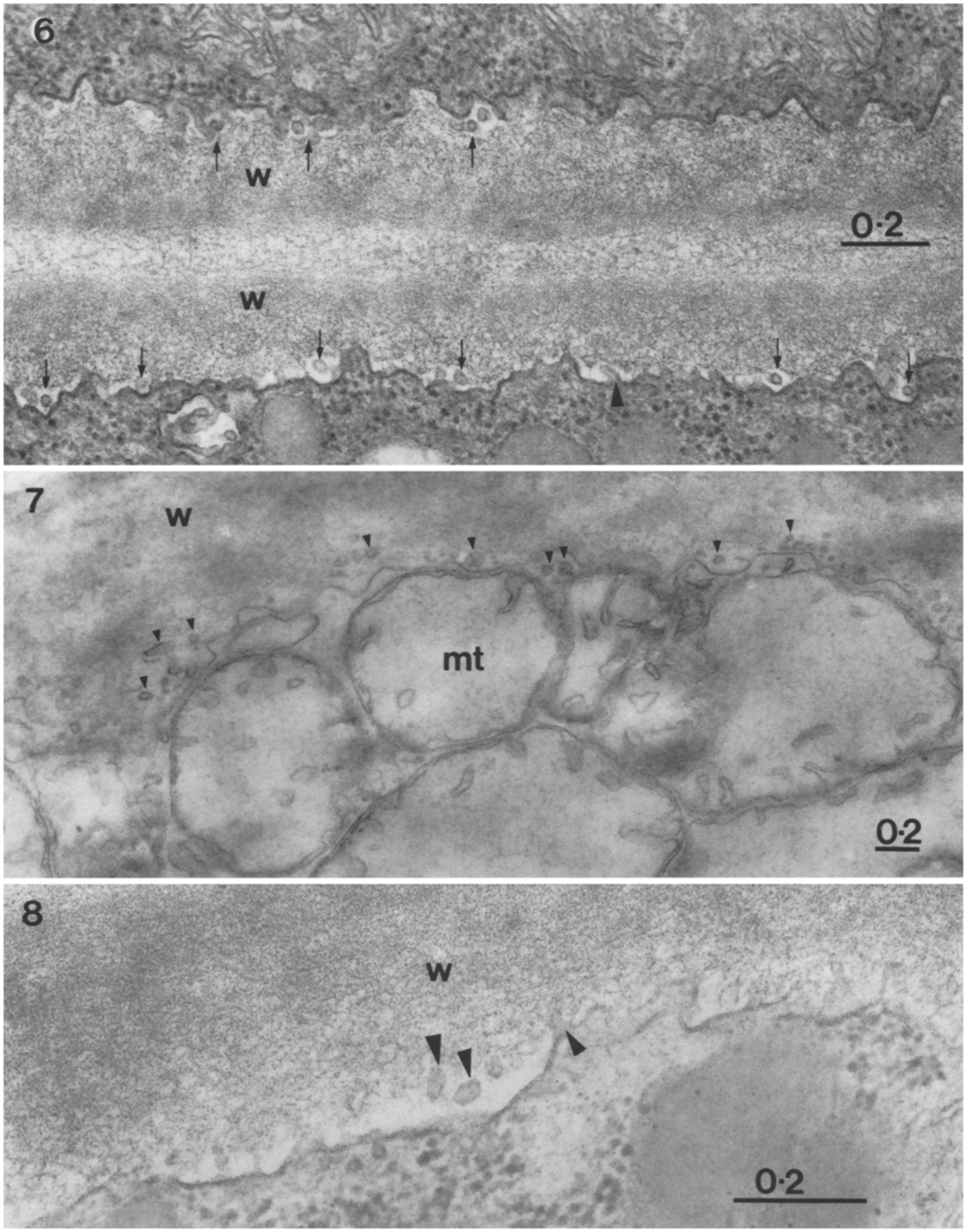


Fig. 6–8. Longitudinal sections through epithelial cells of *Hordeum vulgare* imbibed for 3 d. Tissue post-stained with uranyl acetate and lead citrate (except **Fig. 7**). *mt*, Mitochondrion; *w*, wall. Scale bars in μm. **Fig. 6.** Aldehyde-fixed, osmium post-fixed material showing typical plasmatubule profiles (*arrows*) in the periplasmic spaces of two adjacent cells; *dart* indicates plasmatubule continuity with the plasmalemma. ×72500 **Fig. 7.** Permanganate-fixed material with swollen mitochondria and plasmatubule-like profiles within the periplasmic space (*darts*). ×37000. **Fig. 8.** Osmium-fixed material showing typical plasmatubule profiles (*darts*) within the periplasmic space. ×113400

variety of orientations (Fig. 6; Harris et al. 1982, Fig. 3). The plasmatubules are present as tubular and/or circular profiles approx. 20 nm in diameter which are sometimes seen to be continuous with the plasmalemma and may be branched (Fig. 5).

Osmium fixation. Osmium primary fixation gives an image (Fig. 8) that is, with respect to both general and plasmatubule ultrastructure, similar to that with aldehyde primary fixation.

Permanganate fixation. General cell ultrastructure with potassium permanganate fixation was similar to that seen with the other two aqueous fixations although some differences were noted. The vacuoles frequently exhibited a shrunken, stellate appearance and the mitochondria appeared to be swollen (Fig. 7). Plasmatubule-like structures, however, were visible within the periplasmic space (Fig. 7) but they generally showed a greater range of morphology and diameter than in aldehyde- or osmium-fixed tissue.

Freeze-substitution. Despite a general similarity in ultrastructure to that observed after either aldehyde or osmium fixation (Fig. 2) a number of differences were evident. Overall contrast of freeze-substituted tissue was low, for example intracellular membranes such as those of the vacuoles and endoplasmic reticulum and also the oleosomes were reduced in contrast. The vacuoles, however, whilst showing smoother outlines than those with aqueous fixation, were amorphous structures and without contents comparable to those observed after aldehyde fixation (compare Fig. 2 with Fig. 1). Organelle morphology and ultrastructure was comparable to that seen with aldehyde or osmium fixation although with some reversal of image contrast (Fig. 2). The epithelial cell walls showed two distinct layers: an inner compact region and a looser, outer fibrillar region. The plasmalemma was generally smoother in appearance than after aqueous fixation but plasmatubules were still present within the periplasmic space (Figs. 3–5). Because of the reduction in the periplasmic space plasmatubules were best observed when the wall-plasmalemma interface was caught in glancing section (Fig. 3). Plasmatubule morphology and structure (Figs. 4, 5) were similar to those recorded for aldehyde and osmium fixation.

Discussion

Glauert (1975) wrote 'since some structures observed in the electron micrographs of ultrathin sections cannot be visualised by any other method there is an inevitable element of subjectivity in judging the quality of the preservation achieved'. Plasmatubules fall into the category of structures observable only in the electron microscope. The subjective element in interpretation may be reduced and the validity of the structure implied if 'a structure (has) the same general appearance after fixation with a number of different fixatives' (Glauert 1975). For most ultrastructural work this usually involves a comparison of images from a range of aqueous fixtives, such as glutaraldehyde-formaldehyde, osmium tetroxide and potassium permanganate, with those images resulting from freeze-fixation of specimens followed by their fracture or ultramicrotomy with or without substitution (e.g. Marchant and Moore 1973). Similarity of structure after different fixations is taken as evidence of structural validity since, for example, 'it seems incredible that two substances so diverse in their chemical reactions as osmium tetroxide and formaldehyde should change the fine structure in essentially the same way as to present an altogether misleading picture of what was present during life' (Baker 1965). The structure of the plasmatubules of scutellar epithelial cells of germinating barley, following fixation with buffered aldehydes and post-fixation with osmium tetroxide, has been described previously (Harris et al. 1982) and confirmed in this paper. Comparison of this image with that obtained after fixation with osmium tetroxide alone shows considerable simility in plasmatubule structure. Fixation with aqueous potassium permanganate, however, gave an image of general cell ultrastructure that was not as satisfactory as either osmium alone or aldehyde-osmium; there was evidence of vacuolar distortion and mitochondrial swelling. Potassium permanganate is used as a fixative where high-contrast, relatively low-power images of endomembrane distribution are required (e.g. Mollenhauer and Morré 1976; Sievers and Heyder-Caspers 1983) but is not considered, in either aqueous or buffered solution, to be a reliable general plant fixative (e.g. Fineran 1970, 1971; Glauert 1975). Our observations support this view with regard to the barley epithelial tissues. However, plasmatubule-like structures were present after potassium permanganate fixation.

Crawley (1965) observed that glutaraldehyde fixation was superior to $KMnO_4$ for the preservation of charasomes. Charasomes are plasmalemmal evaginations in the alga *Chara* and are directly analagous to the plasmatubules of barley. Charasome structure was subsequently confirmed by freeze-etching (Franceschi 1981). With plasmatu-

bules of barley, the use of a range of aqueous fixatives alone to verify their structure may still leave some reservations. An alternative anhydrous primary fixative is rapid freezing of the tissues. In this study we have followed freeze-fixation by acetone substitution and anhydrous osmium fixation prior to embedding and sectioning. Despite the inherent theoretical advantages of freeze-substitution, however, it is doubtful whether freezing is instantaneous and it is very difficult to avoid ice-crystal damage (Harvey 1982). These considerations have tended to limit the succesful botanical application of freeze-substitution to unicellular structures with a high surface area to volume ratio, such as yeast cells (Hereward and Northcote 1972) and fungal hyphae (Howard and Aist 1979). Such biological systems are more amenable to the rapid cooling necessary for good ultrastructural preservation than are the multicellular systems of higher plants. Here too, however, some success has been achieved by, for example, Hereward and Northcote (1972) and Browning and Gunning (1977), although in the latter instance preservation varied from cell to cell within the material examined.

In our freeze-substitution work with barley we have had problems of ultrastructural damage caused by ice-crystal formation. Such damage was, however, confined to sup-epithelial tissues and, in some cases, the innermost 25% of the epithelial cells. The plasmatubule-containing regions of these epithelial cells were unaffected by ice damage and were well preserved. Using freeze-substitution the image obtained was essentially the same as that observed with primary fixation in aldehyde or osmium. Differences were noted, e.g. reduction of the contrast of the membranes of the endoplasmic reticulum and the differentiation of the wall into two distinct layers in freeze-substituted material. Reduction in contrast of the lipid components may be directly related to lipid extraction by the solvent of substitution (compare also Howard and Aist 1979). Despite these differences, plasmatubule structure after freeze-substitution was similar to that observed with either aldehyde or osmium as the primary fixative.

In view of the similarity in ultrastructure shown by both freeze-substitution and a number of aqueous fixatives, we conclude that plasmatubules are not artefacts of the fixation process but real structures present within the scutellar epithelial cells of 3 d-germinated barley. The results above illustrate that, in appropriate circumstances, plasmatubule structure, reproducibly shown by a range of aqueous fixatives, can be confirmed with freezing as the primary fixative. In many multicellular tissue freeze-fixation without cryoprotection is not feasible but we would suggest that plasmatubule structure in such tissues e.g. mung-bean cotyledons (Harris 1981) and pea leaf minor veins (Harris and Chaffey 1985) could be acceptably verified by complementary images from a range of aqueous fixatives.

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