A SUBERIZED LAYER IN THE CELL WALLS OF THE BUNDLE SHEATH OF GRASSES

By T. P. O'Brien* and D. J. Carr†

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Summary

Suberized lamellae occur in all walls of the cells of the mestome sheath of wheat and oat leaves. These lamellae are perforated by plasmodesmata at all pit fields that they share with living cells. In maize, the walls of the parenchyma sheath ("starch sheath") contain similar suberized lamellae. The significance of these observations for solute and water exchange between the mesophyll and the bundle is discussed.

I. Introduction

Ever since Schwendener's (1890) thorough study of more than 100 species the structure of the bundle sheaths in grass leaves has provided important taxonomic characters (see Brown 1958). Schwendener recognized two groups of grasses, those with two bundle sheaths and those with one. In those with one sheath (e.g. the genera *Zea*, *Coix*, *Saccharum*, *Andropogon*, *Setaria*, *Pennisetum*), the cells of the sheath are parenchymatous and commonly develop chloroplasts that may be rich in starch (the "starch sheath"). In those with two sheaths (e.g. the genera *Triticum*, *Avena*, *Hordeum*, *Lolium*), the outer sheath is parenchymatous and often develops chloroplasts, but the inner sheath (termed the mestome sheath) is usually colourless and its cells may develop asymmetrically thickened walls. Schwendener showed that the walls of the mestome sheath in those grasses with two sheaths and the walls of the parenchyma sheath in those with only one sheath contained a layer which was resistant to digestion in concentrated sulphuric acid, a property shared with the Casparian strip of endodermal cell walls in roots.

The Casparian strip of the endodermis of roots has long been interpreted as an impermeable layer that separates the free space of the cortex from that of the stele. Good evidence to support this view is scanty since the chemical composition of the Casparian strip is almost unknown. It is usually regarded as being "suberized", but precisely what this means is not very clear. Suberized walls generally contain lipids of high molecular weight that can be stained with the Sudan dyes, and phenolic substances (perhaps lignin) which stain with safranin (and other cationic dyes) even at low pH. Bonnett (1968) has recently studied the fine structure of the Casparian strip in the endodermis of *Convolvulus* roots. The cell membrane was bound firmly to the wall at the strip, a fact that may be related to the resistance of this layer of membrane to plasmolysis. Apart from this, the suberized layer of the wall showed no significant substructure.

* Botany Department, Monash University, Clayton, Vic. 3168.

† Research School of Biological Sciences, Australian National University, Canberra, A.C.T. 2601.

Other than these observations, little is known about the Casparian strip in roots. In leaves, Trapp (1932) demonstrated that the endodermis in the leaves of certain members of the Plantaginaceae was impermeable to acid dyes and ferric salts. Van Fleet (1950) has shown by a variety of histochemical tests that the mestome sheath of grasses has characteristics in common with the endodermis, including the reactions of the tissue to fat stains, polychrome dyes, and oxidation–reduction indicators. However, in maize, which has only a parenchyma sheath in most of its bundles, van Fleet did *not* find endodermal characteristics, though Schwendener (1890) stated clearly that the walls of the parenchyma sheath in maize were resistant to acid digestion.

Whether the free space of the epidermis and mesophyll in grass leaves is separated from that of the vascular bundles by a suberized layer is of some importance to discussions about solute and water exchange between these tissue systems. In this paper, a layer that is probably the site of suberin deposition is described in the walls of the mestome sheath of wheat and oats and in the parenchyma sheath of maize.

II. Materials and Methods

Triticum aestivum cv. Gabo and *Avena byzantina* cv. Klien 69B were grown in the Canberra phytotron at 2000 f.c. and 80% relative humidity on an 8-hr photoperiod (day temperature 25°C, night temperature 20°C). Tissues were taken from tip, middle, and base of fully expanded second leaves. The samples of maize leaf were taken from the middle of a mature leaf (number 7 from the base) of a plant growing in solution culture. For light microscopy, the specimens were fixed in acrolein and embedded and sectioned at 1–4 μm in glycol methacrylate (Feder and O'Brien 1968). For electron microscopy, the tissues were fixed for 2 hr in 1·5% glutaraldehyde solution buffered at pH 6·8 in 0·025M phosphate buffer. They were then transferred to 6% glutaraldehyde (similarly buffered) for 16 hr, rinsed, post-fixed in 2% OsO_4, dehydrated in methyl cellosolve, and embedded in Araldite. Sections were doubly "stained" with uranyl acetate and lead citrate. Full details of specimen preparation are given by O'Brien (1967). Stained sections, or unstained sections treated with silver hexamine (Rambourg 1967; Pickett-Heaps 1968), were examined in an Hitachi HU 11E electron microscope at 75 kV.

III. Results and Discussion

Figures 1 and 2 show the structure of the bundle and its sheaths in wheat and maize respectively. In wheat (and oats) the bundle is surrounded by a layer of cells, the mestome sheath, whose lignified cell walls are thicker on the side that lies nearer to the conducting elements. Starch is absent from these cells and occurs only as minute grains in the plastids of the mesophyll. External to the mestome sheath is a somewhat irregular layer of cells, the parenchyma sheath, whose chloroplasts are somewhat smaller and less strongly stained than the chloroplasts of the mesophyll. Most of the bundles in the maize leaf are like that shown in Figure 2 (the midrib is

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Fig. 3.—Electron micrograph showing part of a cell of the parenchyma sheath (*ps*), mestome sheath cell (*msc*), and tracheary element (*te*), seen in transverse section in a wheat leaf. The mestome sheath cell is comparable to that seen at the left-hand arrowhead in Figure 1. Note the thin suberized lamella (*sl*) that is present in the walls that separate the mestome sheath cell both from the parenchyma sheath and from the tracheary element. *hw*, hydrolysed wall of tracheary element.

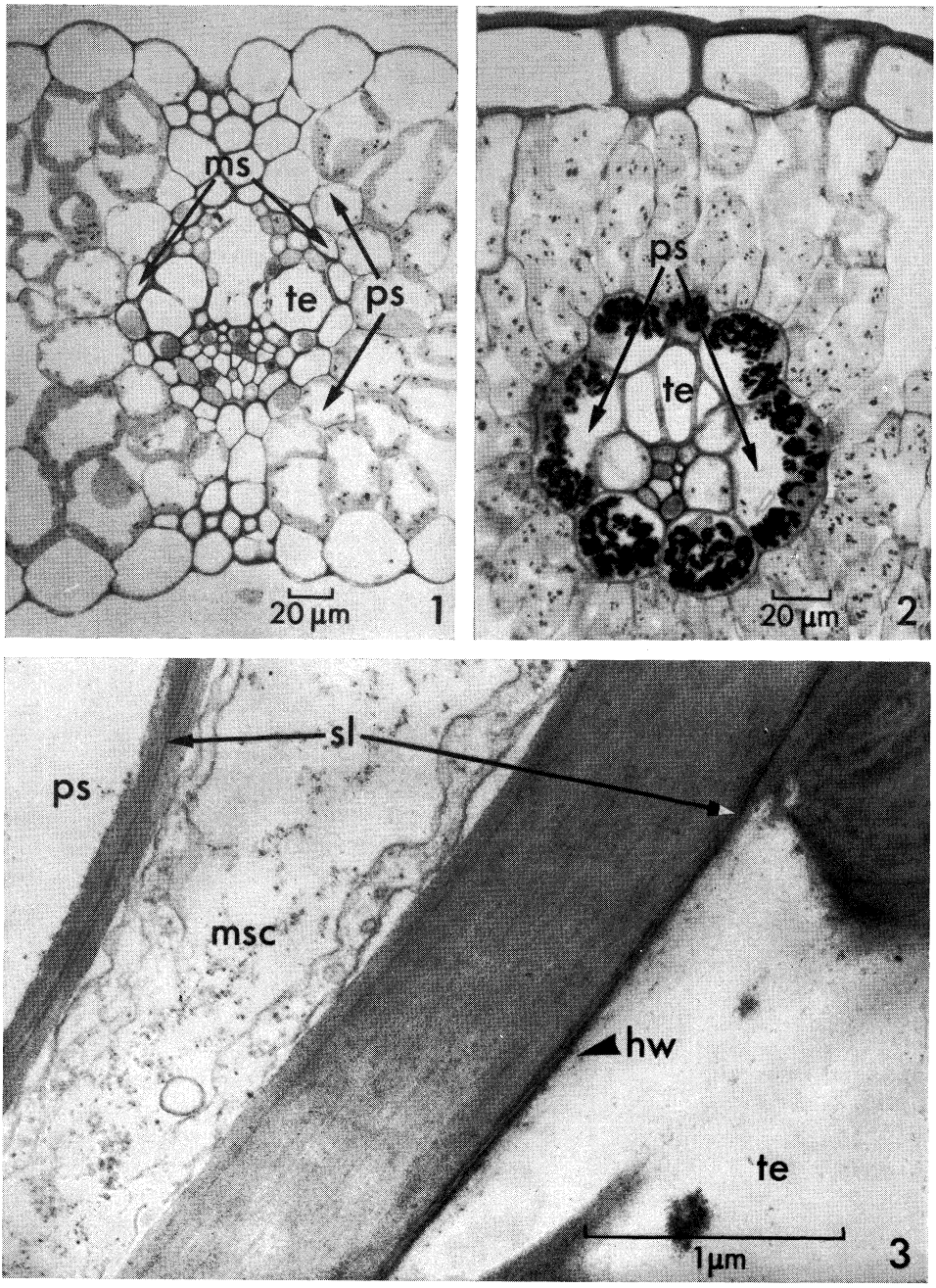

Fig. 1.—Transverse section of wheat leaf showing a vascular bundle, the mestome sheath (*ms*) and parenchyma sheath (*ps*). *te*, tracheary element. Periodic acid–Schiff's reaction, toluidine blue.
Fig. 2.—Transverse section of a small vascular bundle in the leaf of maize. The bundle is enclosed within a parenchyma sheath (*ps*) whose chloroplasts are rich in starch. Note that the plastids tend to lie on the side of the cell that abuts the mesophyll. *te*, tracheary element. Periodic acid–Schiff's reaction, toluidine blue.

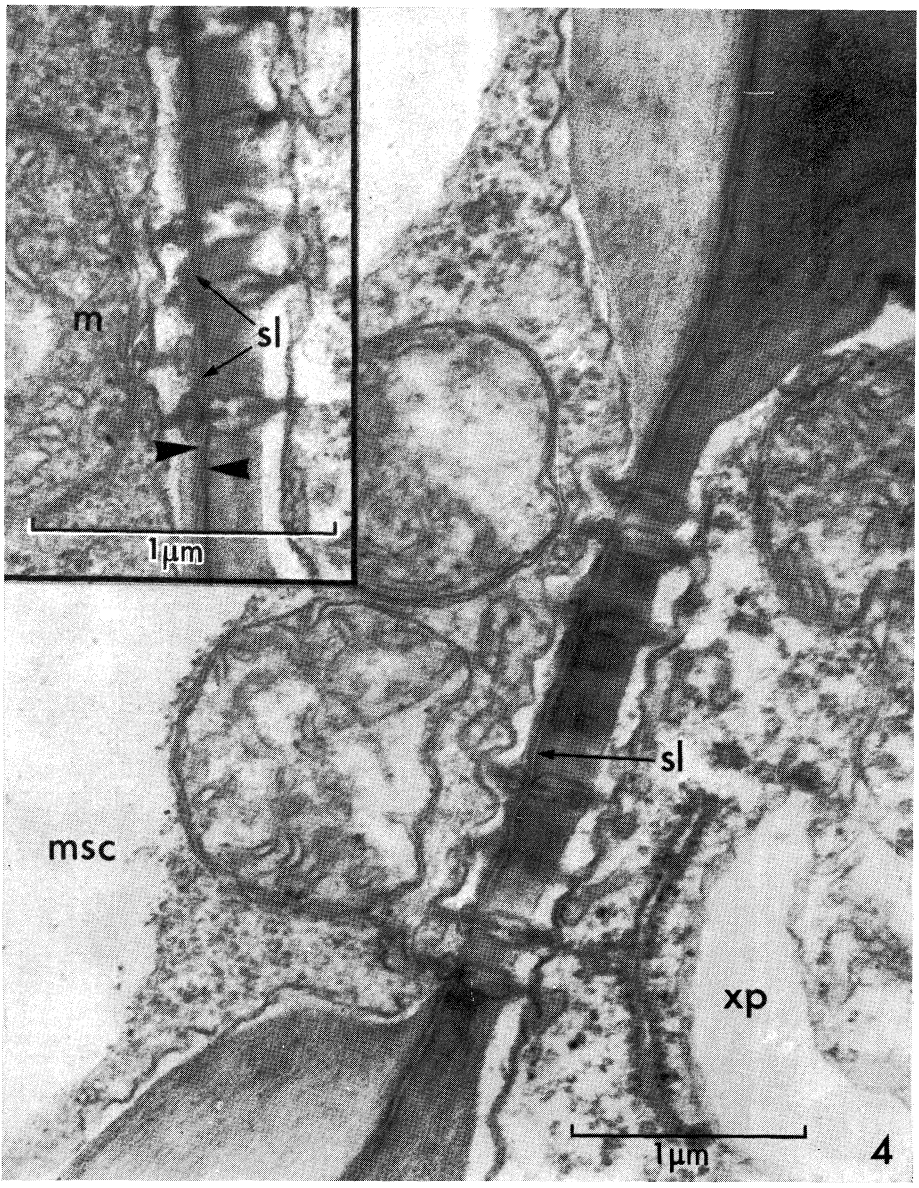

Fig. 4.—Pit field between a mestome sheath cell (*msc*) and a xylem parenchyma cell (*xp*). The suberized lamella (*sl*) is continuous across the pit field, encasing the plasmodesmata that traverse the pit. Two mitochondria lie in the cytoplasm near the pit on the sheath cell side. The inset shows part of a similar pit at higher magnification. The suberized lamella (within which one can detect a number of thinner layers; black arrows) appears to constrict each of the plasmodesmata which have a distinct waist where the suberized lamella is in contact with the membrane that lines the plasmodesmatal canal. *m*, mitochondrion.

rather different in its organization). The bundles are not surrounded by a mestome sheath but rather by a starch sheath, a layer rich in chloroplasts with abundant starch grains. Within these cells, the chloroplasts tend to lie on the side that is next to the mesophyll cells. Esau (1967) comments on a similar distribution of plastids in the bundle sheath cells of the minor veins of *Beta*.

Figure 3 is an electron micrograph which shows part of a mesophyll cell, mestome sheath cell, and tracheary element in wheat. While the preservation of these very mature leaf cells is satisfactory when the tissue is fixed in acrolein and embedded in glycol methacrylate (Fig. 1), collapse of thin walls and marked plasmolysis are all too common when the same tissue is fixed in glutaraldehyde–osmium tetroxide and embedded in Araldite (see also Fig. 5 and O'Brien 1966). Thus, in Figure 3 we see both the thin outer wall of the mestome sheath cell folded inwards where it abuts the mesophyll cell (whose contents are plasmolysed), and the thick inner wall where it abuts the tracheary element. Each of these walls contains a layer which, at this magnification, has a characteristic appearance, two electron-dense lines bounding a region of lower electron density. This "sandwich" structure varies from 25 to 40 nm in width in different parts of the wall of the mestome sheath cells. Near tracheary elements it is most evident where the unlignified primary wall of the tracheary element has been converted to a hydrolysed wall during the closing stages of differentiation of the tracheary element (O'Brien and Thimann 1967*b*; O'Brien 1970). At higher magnification, further substructure is evident within the region of lower electron density, especially within the pit fields (Fig. 4 and inset). The region of low electron density is seen to contain 3–4 thin lamellae (each about 3–5 nm thick) of low electron contrast, separated by varying thicknesses of electron-dense material. Since this multilayered structure is found in wheat and oat leaves only in the walls of the mestome sheath and in maize leaves only in the walls of the parenchyma sheath (see Figs. 8 and 9), it seems likely that this layer is rich in the acid-resistant material demonstrated by Schwendener (1890) in these cells. This is supported also by the presence of lamellae with a similar appearance in other types of walls that are known to be suberized (Sitte 1962; de Vries 1968; Wattendorf 1969). A similar layer, extremely electron-dense after permanganate fixation, was illustrated in the endodermis of maize roots (Leech, Mollenhauer, and Whaley 1963) but they did not comment on its significance. For convenience we have called this unusual multilayered structure in these walls a "suberized lamella". This is intended as a descriptive term and does not imply knowledge of the location of any of the components of suberin within the layer.

Numerous plasmodesmata connect the cells of the mestome sheath with those of the parenchyma sheath and with vascular parenchyma cells (Fig. 4 and inset). In most respects, the plasmodesmata appear to be identical to those illustrated in the cells of oat coleoptiles (O'Brien and Thimann 1967*a*, 1967*b*). However, the presence of the suberized lamella modifies the structure of the plasmodesmata in one important respect. Each plasmodesma appears to be constricted by the suberized lamella so that the membrane that lines the plasmodesmatal canal is pressed against the "desmotubule" (Robards 1968), the electron-dense core that occupies part of the lumen of the canal. This encroachment of the suberized lamella upon the plasmodesmata, such that they show a distinct waist, is most evident in the inset to Figure

4. This modification of the structure of plasmodesmata occurs in all pits that are traversed by suberized lamellae, including the pits between the cells of the parenchyma sheath in maize (Fig. 9). If the plasmodesmatal canal is important in symplastic transport through plasmodesmata, the suberized lamellae must modify the role of the canal in these plasmodesmata.

Mitochondria often lie in the cytoplasm within the pits of the mestome sheath cells (Fig. 4 and inset). While this association is not seen in every section through a pit field, it occurs frequently and does suggest that the mitochondria may be providing energy for solute transfer at the pit fields.

Careful examination of many sections of both wheat and oat leaves has shown that the cells of the mestome sheath are invariably encased completely within a suberized lamella identical to that illustrated in Figures 3, 4, and the inset to Figure 4. Further evidence to support this point comes from study of the reaction of these suberized walls with silver hexamine. Pickett-Heaps (1968) pointed out that lignified walls of tracheary elements in wheat roots reacted strongly with the silver hexamine stain without prior treatment with periodate, a reaction that is probably related to the well-known Schiff positivity of many lignified walls (see Jensen 1962, p. 190). In this study it was possible to confirm that the lignified walls of tracheary elements, fibres, and guard cells reacted with the silver hexamine reagent without periodate oxidation. In addition, it was found that, under the same conditions, the suberized lamellae of the mestome sheath were even more reactive than lignified walls. The chemical basis of this reaction is not clear. Neither the lignin reaction nor the suberin reaction could be prevented by prolonged (up to 60 hr) blockade in 2,4-dinitrophenylhydrazine or by oxidation with 0·2M chlorous acid at room temperature (Rappay and van Duijn 1965). The lignin and suberin reactions do not depend upon the glutaraldehyde–osmium tetroxide fixation for these reactions occur equally well in specimens fixed solely in ethanol and embedded in Araldite after soaking in propylene oxide.

Whatever the chemical basis of this reaction of lignin and suberin with silver hexamine may be, it was most useful in the present context as a check upon the continuity of the suberized lamellae. Figure 5 and inset show an area of the mestome sheath of wheat reacted with silver hexamine at 60°C for about 15 min. The section had not been treated with periodate and the reaction was stopped before the lignified walls had developed their full stain. One may trace a continuous heavy deposit of silver in all walls of the mestome sheath. The position of this deposit of silver corresponds to the position of the unusual layer shown in Figures 3 and 4 and is part of the evidence that this layer is suberized.

The appearance of the pit fields, especially in slightly oblique views of sections stained with silver hexamine (Fig. 5, inset), confirms that the plasmodesmata of each pit field are completely encased by the suberized lamella. One may readily detect

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sheath cells are in contact. Though nothing can be deduced about the nature of this silver-depositing reaction, it is useful for it shows that the sheath cells are completely encased in suberin. *p*, plastid. The inset shows an oblique view of a pit field between a mestome sheath cell (*msc*) and a phloem parenchyma cell (*pc*) in a section treated as for Figure 5. Clearly, the suberized lamella (white-tipped black arrows) completely encases the plasmodesmata (small white arrow).

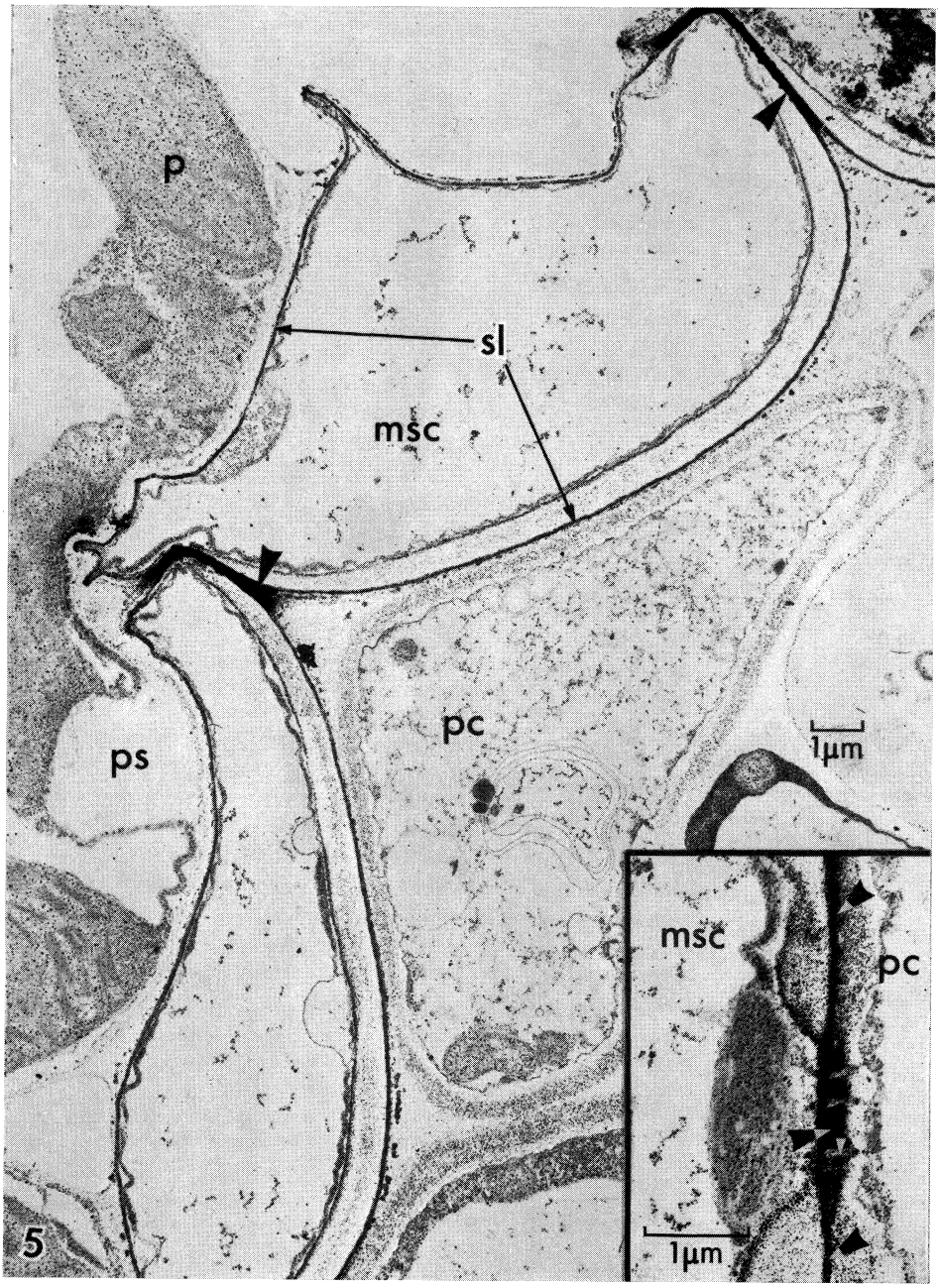


Fig. 5.—Electron micrograph of a transverse section of a wheat leaf, showing part of a cell of the parenchyma sheath (*ps*), mestome sheath cell (*msc*), and phloem parenchyma cell (*pc*). The section was treated with silver hexamine solution in the absence of periodate, and is otherwise unstained. The suberized lamella (*sl*) reacts strongly with silver hexamine, depositing silver in all walls that contain the lamella. In addition, there is a heavy deposit (arrows) in the radial walls where two

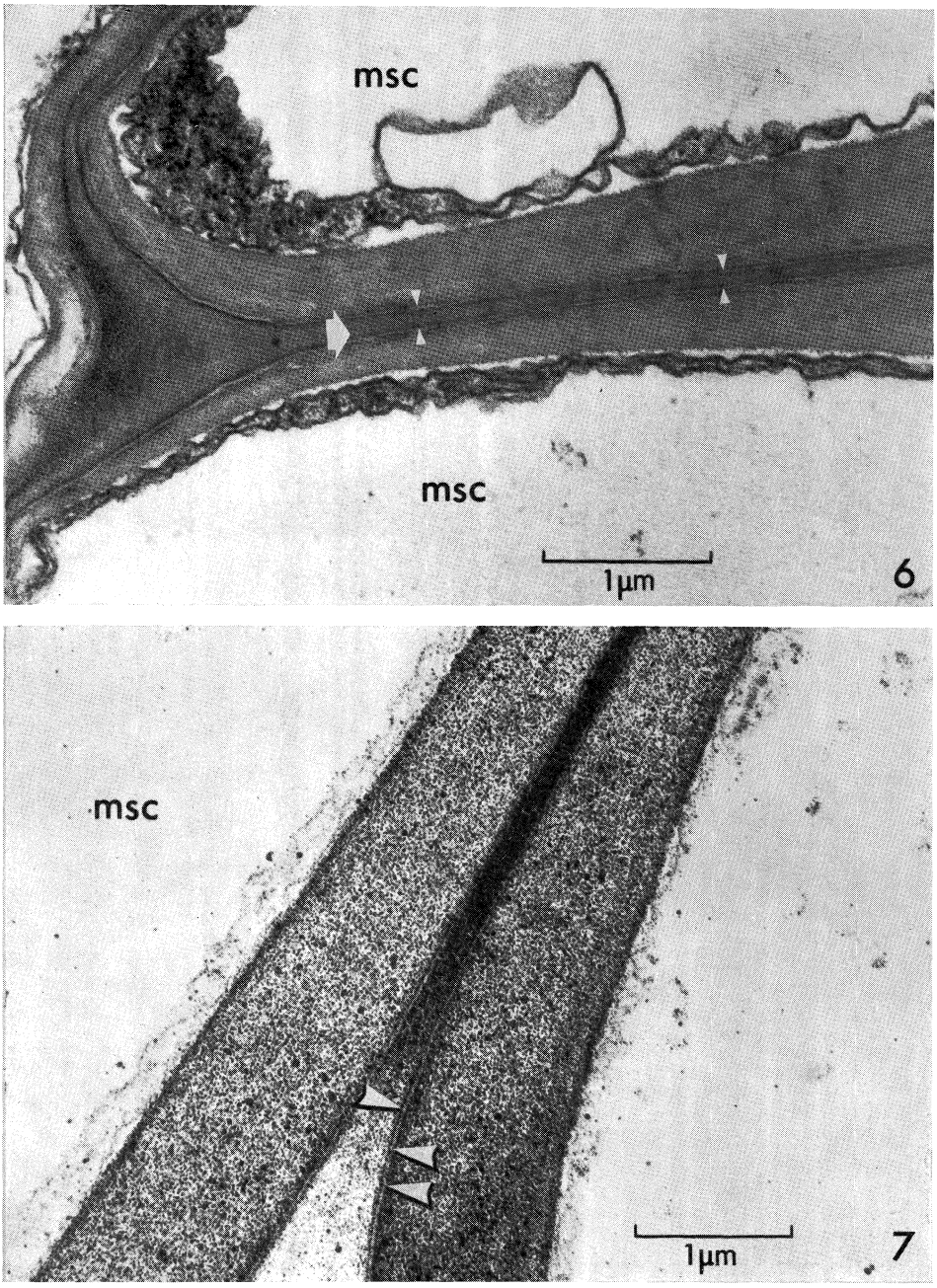
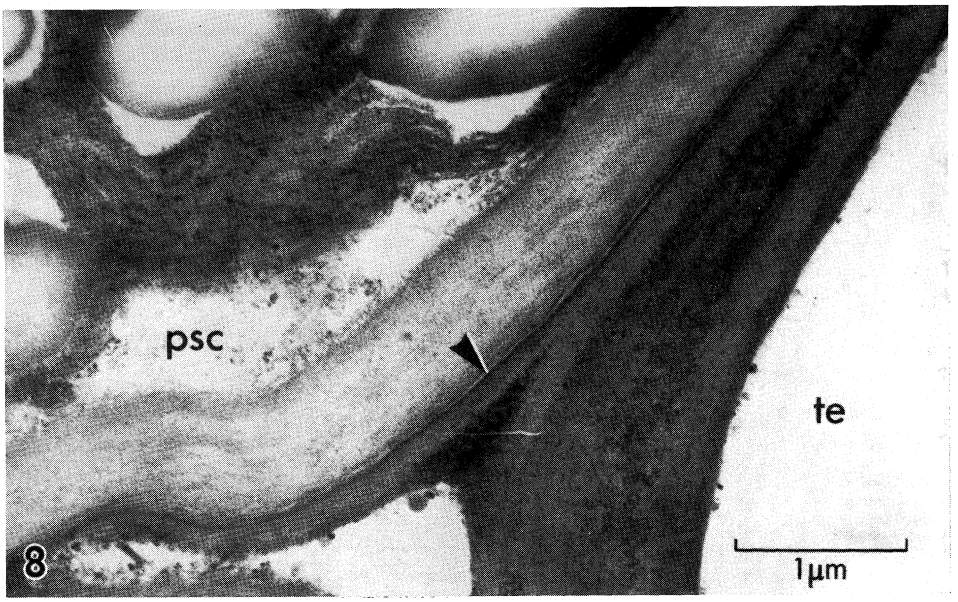


Fig. 6.—Electron micrograph of a transverse section of a wheat leaf showing the junction between the radial walls of two mestome sheath cells (*msc*) and an adjacent cell of the parenchyma sheath. The suberized lamella in the wall of each of the mestome sheath cells is evident at the small white arrows. The layer of wall which lies between these suberized lamellae to the right-hand side of the large white arrow corresponds to the very electron-dense regions labelled with large arrows in Figure 5. A similar region exists in the corresponding zone of Figure 7.



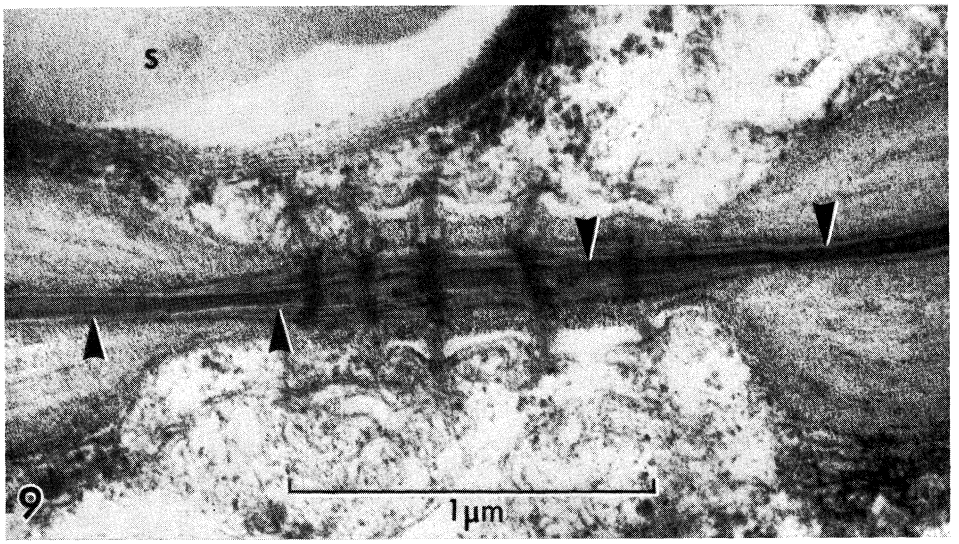


Fig. 8.—Electron micrograph of the junction between a parenchyma sheath cell (*psc*) and a tracheary element (*te*) in the leaf of maize. A suberized lamella is evident at the arrow.
Fig. 9.—A pit field between two cells of the parenchyma sheath in maize. Two suberized lamellae can be seen in the radial walls and are continuous with two thicker suberized lamellae (large arrows) between the plasmodesmata in the pit field itself. *s*, starch.

Fig. 7.—Electron micrograph of a tranverse section of an oat leaf showing part of the radial wall of two mestome sheath cells (*msc*). The section has been treated with silver hexamine reagent (but without prior periodate oxidation), and is otherwise unstained. The position of the suberized lamellae is marked by a deposit of silver (arrow) that is as dense as the deposit in the lignified walls. The electron-transparent region of the suberized lamellae visible in Figures 3, 4, and 6 appears to correspond to the narrow strip of reduced silver deposition that lies between the dense deposits marked by the arrows. A heavy silver deposit also occurs in the layer of wall between the suberized lamellae, confirming that this silver-depositing material is present in the radial walls of both oats and wheat (see Fig. 5).

the unstained plasmodesmatal canal of each plasmodesma surrounded by a continuous deposit of silver.

The reactions of the radial walls of the mestome sheath cells deserve special comment. In "standard" sections (Fig. 6), a slightly enhanced electron contrast is the only distinguishing feature of that region of the primary wall that lies between the two suberized lamellae in the radial walls. However, in both wheat (Fig. 5) and oats (Fig. 7), this region is seen to react very strongly with silver hexamine reagent even when the sections have not been treated with periodate. Although it is impossible to say which component(s) of the suberized lamella is responsible for the silver hexamine reaction, close inspection of Figure 7 suggests that the electron-transparent layers of the suberized lamellae seen in Figures 3 and 4 correspond to regions of reduced silver deposition in Figure 7. The strong reaction in the radial wall of the mestome sheath cells suggests that some component in the electron-dense layers of the suberized lamellae of Figures 3 and 4 is present throughout this part of the primary layer of the radial wall. Presumably, it is this material which is responsible for the enhanced electron contrast of this region in Figure 6. It will be interesting to determine the reactions of the Casparian strip in roots to silver hexamine reagent under these conditions because the appearance of the radial walls in Figure 6 is closely similar to the appearance of the Casparian strip in *Convolvulus* roots (Bonnett 1968).

Figure 8 shows the junction between a cell of the starch sheath and a tracheary element in maize. A suberized lamella is very clearly evident between the two cells. Figure 9 shows the radial walls and connecting pit between two cells of the starch sheath. On either side of the pit, one may readily detect the two thin layers of suberized lamellae. The plasmodesmata (which are slightly wrinkled in this section) are encased in suberized layers even thicker than those that occur in comparable pits in cells of the mestome sheath of wheat and oats.

These mature leaves of maize have proved to be exceedingly difficult to embed and it has not yet been possible to examine what happens in their midrib whose vascular bundle possesses a remnant of the mestome sheath. The massive deposition of starch in the plastids of the starch sheath, coupled with the presence of lignin and silica in the epidermal cells, leads to serious disintegration of the sections during examination in the electron microscope. Nevertheless, it was possible to confirm the presence of thin suberized lamellae in the walls of all cells of the starch sheaths of small vascular bundles. They are also illustrated, though ignored, in the sheath cell walls of sugar-cane (Laetsch and Price 1969). Curiously, a suberized lamella sometimes seemed to be absent from parts of the junction between tracheary elements and starch sheath in maize, even when such a lamella was clearly discernible in the radial walls and at the mesophyll–sheath junction of the same cell. Until better sections can be produced, one must leave open the possibility that a complete suberized lamella does not separate the tracheary elements from the sheath cells of maize.

IV. Concluding Remarks

Nearly 80 years ago, Schwendener, armed only with concentrated sulphuric acid as an agent of selective destruction, deduced correctly that the parenchyma sheath of maize contained suberized lamellae similar to those of the mestome sheath

of other grasses. This is an astonishing fact, a tribute to his patience and to the quality of the objectives of that period, and a sobering lesson for the more vocal proponents of the idea that light microscopy is outmoded.

The significance of the observations reported here depends a good deal on just what kind of permeability barrier the suberized lamellae provide. Preliminary experiments with moderately concentrated solutions of alcian blue (0·1–1%), fast green FCF (0·01–0·5%), and acid fuchsin (1%) have shown that these dyes are rapidly transported in the transpiration stream of freshly cut wheat leaves. Transverse sections of fresh material from such leaves show that, initially, the dyes are confined to the bundles and show no tendency to penetrate beyond the suberized walls of the sheath cells, though they spread easily through the non-suberized walls of vascular cells. However, the leaves have always wilted within 15–30 min of isolation and sections of such wilted leaves may show dye either still confined by the sheath or spreading into walls outside the sheath *and into the contents of the sheath cells*. This suggests that the sheath cells have been killed, and dye is simply leaking through the killed cells. Aqueous solutions of basic fuchsin (0·5%) also spread laterally from the bundle, staining the subepidermal fibres within a few minutes of reaching any particular level in the leaf. Attempts to use truly vital, fluorescent dyes at high dilution (e.g. fluorescein) have so far met with failure due to the marked native fluorescence of the walls of the mestome sheath cells. In any event dye molecules are often large compared to the types of solute (sugars, salts, amino acids, hormones) in whose transfer one is really interested. It is not an easy task to design experiments to test the permeability of the suberized lamella to these substances. The use of selective inhibitors of solute transport, inhibitors that are not in themselves excessively toxic, may be a fruitful approach.

One cannot present any facts on the permeability of these suberized lamellae to water for this has never been measured. However, suberin is generally believed to be somewhat similar to cutin, the component of the cuticle which, especially when impregnated with wax, seems to be responsible for the resistance of the epidermal cells to water loss. Certainly these suberized walls stain with sudan black B as does the cuticle of the same leaf. If suberized lamellae should prove to be relatively impermeable to water, one would have to face the possibility that, at least in the leaves of some Gramineae, Cyperaceae, and Juncaceae, water loss is regulated at the vascular bundle as well as by the stomata. Some control of water loss at the vascular bundle might be essential if an adequate supply of water is to be maintained to the leaf tip. This might be especially important in these long leaves under conditions of water stress when the lower half of a long linear leaf might well be able to transpire all of the water available to the leaf. If the suberized lamellae restrict passive loss of water from the free space of the bundle to that of the mesophyll, forcing the water to follow a symplastic route through the sheath cells, the water flow across the sheath could be regulated by the vital activity of the sheath cells.

There is at the present time a good deal of interest in "transfer cells" (cells whose labyrinthine walls produce increased area of cell membrane) for it is believed that these cells help to mediate solute exchange between tissue systems [see e.g. Gunning, Pate, and Briarty (1968) and the references contained therein]. In an extensive survey of the distribution of transfer cells in the leaves of angiosperms,

Pate and Gunning (1969) have shown that these cells are absent from the leaves of most monocotyledons, including grasses. Their absence from grass leaves is not due to any inherent inability of grasses to form them for they have been illustrated in the megagametophyte of maize (Diboll 1968), in the epithelium of emerging root primordia of maize (O'Brien and McCully 1969, fig. 50), and in the plexus of vascular tissue at the scutellar node of germinating wheat seedlings (O'Brien and Swift, unpublished data). It is possible that transfer cells are absent from grass leaves because of the presence of suberized lamellae in the walls of the bundle sheath cells. Transfer cells in leaf veins are thought to assist exchange between xylem and phloem, and between both of these systems and the adjacent mesophyll. If passive loss of solute is restricted from the vascular bundle because of the suberized lamellae, and if the bulk of solute transfer between bundle and mesophyll must go *through* the plasmodesmata rather than across the free space, transfer cells would be unnecessary.

Finally if the plasmodesmata of these cells are special sites of solute transfer, it should be possible to demonstrate the presence of solute-dependent ATPase activity at these regions.

V. Acknowledgments

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