Electron Microscopy of Ultrathin Frozen Sections of Pollen Grains

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RELIABLE ANALYSIS of the ultrastructure of cells and tissues by means of the high resolving power (ideally of about 10 A [*1*]) of the electron microscope can only be carried out on extremely thin preparations—i.e., below 0.2 μ in thickness. Most previous investigations of sections of plant cells with the electron microscope (*2–7*) were performed on material embedded in celloidin-paraffin, in paraffin-wax, or in methacrylate plastic, and then usually sectioned thinly according to the techniques developed by Pease and Baker (*8*) or by Newman, Borysko, and Swerdlow (*9*). Embedding of such cells involves the usual dehydration and infiltration with organic solvents, followed by impregnation with the aforementioned embedding media—procedures that can obscure fine detail in the sections. The removal of the embedding media from the thin sections can also introduce change or distortion (*10*).

One of us (Fernández-Morán, *11–13*) has developed a method for obtaining ultrathin frozen sections of unembedded, fixed material without preliminary treatment. In a number of cases where comparison with collodion-paraffin– and methacrylate–embedded sections was possible, especially in the study of nerve fiber ultrastructure and of bacteria sections (*14*), the frozen sections have proved either equal or superior to the embedded material in revealing delicate structural details. Parallel studies with phase contrast microscopy can also be readily made with the frozen sections.

The present communication summarizes the application of this freezing, sectioning technique to a preliminary study of the mature pollen grain wall.

Pollen of *Tradescantia paludosa* E. Anders. & R. E. Woodson (University of Minnesota, Clonal Line 6–50) was utilized. The mature pollen grains from recently dehisced anthers were fixed in freshly prepared Navashin-Karpechenko[2] and Champy (*15*) fluids for 12–24 hours. The fixation vials were evacuated for 2–5 minutes after the introduction of the pollen material and were maintained at refrigerator temperatures. The fixed pollen grains were washed in distilled water and were then collected, by centrifugation or filtration, on small disks of filter paper. The disk with the wet sediment of pollen grains was frozen on the special stage of the freezing microtome assembly (*12*) attached to the specimen holder of a Spencer rotary microtome, Model No. 820, equipped with an electric motor drive to deliver 50–100 rpm. The well-frozen, fixed pollen was then advanced until it came in contact with the knife edge, and several sections, 1 μ in thickness, were cut and floated off on the surface of the water reservoir attached to the knife. Both ordinary (Schick) razor blades fitted in a special knife holder and the excellent glass knives described by Latta and Hartman (16), but prepared by thermal cracking, were used.

Sections thin enough for electron microscopy (0.1–0.2 μ) were then obtained by disengaging the mechanical advancing system of the microtome, thus permitting the continuous thermal expansion of the stage to advance the specimen toward the knife edge by fractions of a micron with each cutting stroke. The sections of the pollen grain spread out immediately upon hitting the water surface, forming a practically invisible thin film. The sections were picked up directly by lowering collodion-covered electron microscope grids parallel to the water surface until they touched the sections, which immediately adhered to them. The sections can also be picked up on glass cover slips coated with a thin collodion film, which can be peeled off afterward for electron microscopy. The latter method is more convenient for examination of the sections with the phase contrast microscope (Zeiss). The sections may be either frozen-dried by placing the grids on a brass block cooled to −50° Celsius or air-dried. The block was transferred to a vacuum evaporation chamber, placed on insulating glass plates, and a vacuum drawn. The sections may then be shadowed with platinum/palladium and reinforced with a vertically deposited aluminum film.

Knife-tilt, meniscus level of the collecting reservoir, consistency of the specimen, variable mechanical factors in the microtome, and motor drive are all critical factors. The method is actually simple and gives dependable results in routine practice. An RCA type EMU electron microscope with a saturated emission type electron gun (accelerating potential, 50 kv), and equipped with RCA telescopic image viewing device and a compensated objective, was used throughout this study.

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[2] Ten ml 1% chromic acid, 4 ml 16% formaldehyde, 1 ml propionic acid.

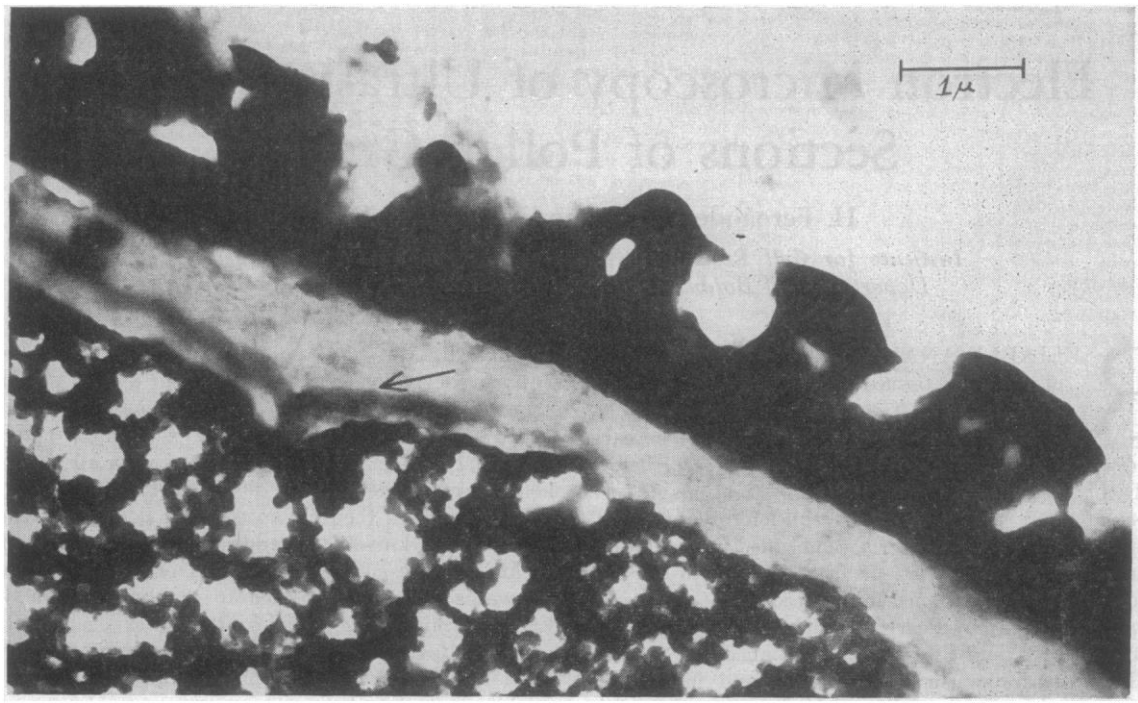

FIG. 1. *Tradescantia*: Wall and contiguous protoplast of mature pollen grain. Frozen section of unembedded, fixed (Navashin-Karpechenko) material; thickness, *ca.* 0.2 μ. Electron micrograph: RCA EMU electron microscope. 50 kv. × 16,000.

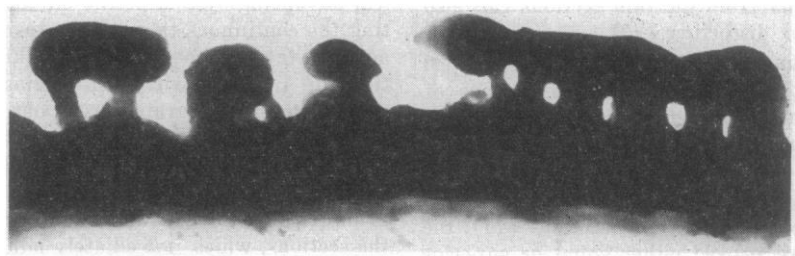

FIG. 2. Same as Fig. 1, except thickness of section, *ca.* 0.1 μ. Portion of exine showing columellate-tectate structure.

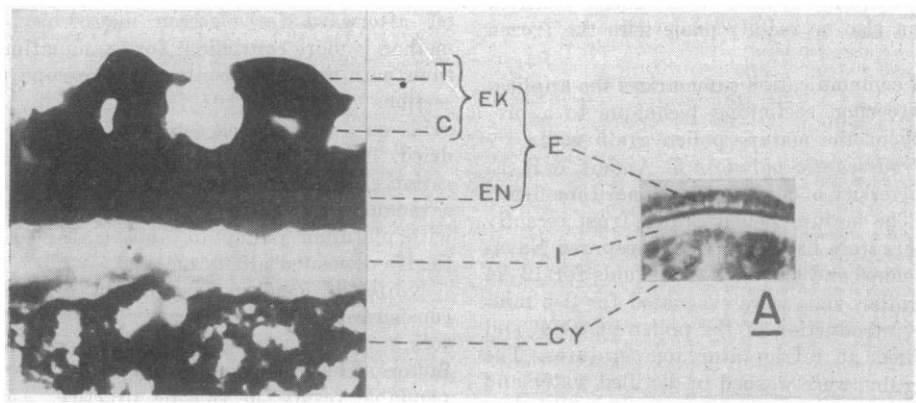

FIG. 3. Summary of structural relationship of components of mature pollen grain of *Tradescantia*: tectum (*T*), columellae (*C*), ektexine (*EK*), endexine (*EN*), exine (*E*), intine (*I*), and cytoplasm (*CY*). *A*, Phase contrast (Zeiss) picture. 1 μ, frozen section of unembedded, fixed material. × 1800.

There are conflicting reports in the literature concerning the relative merits of various fixatives for electron microscopy (*cf. 4, 17–20*). In the limited study reported here, both fixatives were similar in

preserving details of the pollen grain wall. Champy fixation, however, demonstrated a much finer texture in the cytoplasm than did Navashin-Karpechenko fixation, although some variation in texture was evident within the protoplast.

Most pollen grains possess a complex wall which, on the basis of visual light microscopy, has been divided into an outer layer or zone, the exine, which itself may be further subdivided; and an inner layer, the intine. The most illuminating recent studies of sectioned exine are those of Christensen (*21*) and Iversen and Troels-Smith (*22*), which are based upon visual light microscopy of thin (1 μ) sections of acetolyzed pollen grains. The present findings are in harmony with these reports. The exine and intine of the *Tradescantia* pollen grains are vividly differentiated by electron microscopy (Fig. 1). The exine is a highly resistant layer, heavily charged with a variety of largely unknown compounds that apparently render the exine very opaque to electrons. Thus in Figs. 1–3 the exine appears dark in contrast to the light intine. As viewed in transverse section, the only exine components resolved by electron microscopy are the discrete columellae, the smallest of which observed are about 0.12 μ in diameter. Their mean diameter is *ca.* 0.2 μ. Usually adjacent columellae are coalescent at their more or less enlarged tips, giving rise to groups of variable lateral extent (Fig. 2). In transverse section the effect is that of a canopy, or tectum, supported by the columellae. In the case of *Tradescantia* this tectum exhibits an irregular discontinuity (Figs. 1, 2), which accounts for the areolate or rugulate character of the exine as observed in surface view with visual light microscopy. The total radial extent (i.e., thickness) of the entire exine layer averages 1.43 μ. This layer is subdivisible into a centripetal layer *ca.* 0.62 μ in thickness, the endexine, from which the columellae extend centrifugally. The discrete columellae have a radial extent (i.e., length) of *ca.* 0.40 μ and the tectum is *ca.* 0.41 μ in thickness. Columellae and tectum together represent the ektexine (Fig. 3).

The intine, a layer *ca.* 0.79 μ in thickness, is readily penetrated by electrons. It lacks the highly resistant properties characteristic of exine. A relationship of fundamental importance is that existing between the wall of the pollen grain and the protoplast. In terms of physical intimacy this may be expressed as the relationship between intine and cytoplasm. Inspection of the original electron micrographs suggests that the intine of the *Tradescantia* pollen grain is a structure which is penetrated by irregular, rather sinuous strands that appear to be intimately associated with peripheral cytoplasm (note arrow in Fig. 1). In some preparations, the intine has the appearance of a complex system of channels permeating a relatively smooth matrix. The most massive of the strands noted have a diameter of about 0.2 μ.

The above interpretation of intine structure is being followed ontogenetically. In totally different examples, such as the growing wall of the alga *Valonia*, it has been suggested that there is no sharp differentiation between protoplast and wall, and that a protoplasmic component must permeate the latter (*23*). Preston and Wardrop (*24*) have suggested the possibility that in cambial cells there is a ramifying relationship between cytoplasm and the growing wall. An intimate connection between protoplasm and the developing layers of plant cell walls has also been postulated by Mühlethaler (*25*).

The technique described above demonstrates in the pollen grain of *Tradescantia* the columellate, tectate type of exine which has been clearly illustrated with visual light microscopy in a number of dicotyledonous species (*21*). The columellae grade down in diameter to *ca.* 0.1 μ, with a minimum intercollumellar distance of *ca.* 0.06 μ. In the case of the intine, the possibility of an intimate, interpenetrating association with cytoplasm is suggested.

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