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Quantitative Freeze-Fracture Study of Plasmalemma and Nuclear Envelope of *Zea mays* Root Cells during Early Germination

M. CRÈVECOEUR, R. DELTOUR, AND R. BRONCHART

University of Liège, Department of Botany, Sart Tilman, B22, B4000 Liège, Belgium

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Descriptive and quantitative observations of the plasmalemma and nuclear envelope of freeze-fractured root cells are reported for quiescent and early germinating maize embryos.

The numerical density of particles exposed on the extracellular fracture faces of the plasmalemma increases significantly between 12 and 24 hr of germination while it remains unchanged on the protoplasmic fracture faces. The plasmalemma is significantly thicker after 24 hr of germination probably due partially to its enrichment in particles.

Between 24 and 72 hr of germination there is a significant increase in the density of nucleopores. The fine structure of the nucleopores changes between 4 and 24 hr of germination. The first 24 hr of germination are also characterized by a spectacular rise in the density of particles of the two nuclear membranes.

The results are discussed with respect to germination, particularly that of the maize embryo in which several cellular and molecular aspects have already been studied.

During early germination of seeds of higher plants, there is a rapid and strong increase of metabolic processes in embryonic cells (Miège, 1975; Mayer and Shain, 1974). This metabolic reactivation leads to the resumption of mitotic activity, growth, and differentiation of the embryo. In several species the ultrastructural changes accompanying this strong metabolic increase have been described (Yoo, 1970; Hallam *et al.*, 1972; Deltour *et al.*, 1979; Sargent and Osborne, 1980). Although modifications in cellular membranes may play a considerable role in controlling germination (Mayer and Shain, 1974), little attention has been paid to changes in their properties and their ultrastructure during germination. Studies have shown that the increase of embryo respiratory activity is linked to the formation of mitochondrial cristae (Nawa and Asahi, 1971; Solomos *et al.*, 1972). The plasmalemma is implicated in the selective transport of water, ions, and other solutes (Oxender, 1972; Lee, 1975) and is the site of several enzymatic activities (Lai and Thompson, 1972; Galbraith and Northcote, 1977). Physiological studies show a rapid modification of plasmalemma permeability in early stages of germination (Simon and Raja Harum, 1972; Parrish and Leopold, 1977; Simon, 1978) and an activation of associated enzymatic activities has been demonstrated cytochemically (Colson, 1979; Deltour *et al.*, 1981). These results suggest that ultrastructural changes most probably occur in the plasmalemma during early germination. Description of the fine structure of this membrane at the time of hydration of seeds is scarce, however (Buttrose, 1971).

On the other hand, the nuclear envelope is the site of various enzymatic activities (Franke, 1974; Harris, 1978; Zbarsky, 1978) and is perforated by nucleopores which have been extensively studied (Franke, 1974; Maul, 1977). Several functions have been attributed to these structures: site of exchange of materials between the nucleoplasm and the cytoplasm (Merriam, 1962; Mepham and Lane, 1969) especially of

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RNP (Stevens and Swift, 1966; Franke and Scheer, 1970; Franke, 1974), site of polyribosome assembly (Mepham and Lane, 1969; Jacob and Danieli, 1972), and site of initiation of DNA replication (DuPraw, 1965; Maul *et al.*, 1972). The sequential reactivation of DNA, RNA, and protein synthesis that occurs during early germination could therefore be linked to ultrastructural changes of the nuclear envelope at this time. The nuclear envelope has been described in a study of the cotyledons of germinating *Cucurbita maxima*, but unfortunately this study starts after 1 day of germination (Lott and Vollmer, 1975).

Thus, the purpose of the present study is to describe, by means of the freeze-fracture technique, the plasmalemma and the nuclear envelope of root cells of the maize embryo through early stages of germination. Changes in thickness of the plasmalemma have also been determined by microdensitometry.

MATERIAL AND METHODS

Freeze-Fracture

Zea mays kernels var CiV2 were germinated at 16°C in an incubator. Radicle tips were excised from dry ungerminated embryos and from embryos at 4, 12, 24, 48 and 72 hr after imbibition; all were fixed for 1 hr in glutaraldehyde (pH 7) at 4°C. Following fixation the embryos were washed in cacodylate buffer. The first millimeter of the radicle tips was then cut off and impregnated for 3 hr with glycerol. For dry embryos and embryos germinated 4 and 12 hr the concentrations of both glutaraldehyde and glycerol were 50% (v/v) to avoid as much as possible a change of tissue water content which was 12, 25 and 45%, respectively (Crèvecoeur *et al.*, 1976). For longer periods of germination, glutaraldehyde 4% and glycerol 25% (v/v) were used.

The radicle tips were transferred to golden specimen holders and frozen in liquid propane (−186°C). Freeze-fracture was done under high vacuum (2 to 3.10^{-6} torr) in a Balzers apparatus (BA 360M). During fracturing the temperature of the samples was maintained at −100°C and the knife was held at −196°C. The radicles were shadowed with platinum for 8 sec at an angle of 45° and then strengthened by deposition of carbon for 10 sec.

Replicas were washed for 24 hr in 75% H_2SO_4 to remove adherent root tips. They were cleaned in twice-distilled water and then in a commercial solution of calcium hypochlorite ("eau de javel") for 12 hr. They were rinsed several times in twice-distilled water before being collected on Formvar-coated copper grids and examined in a Siemens Elmiskop 101.

Quantitative Study of Membrane Fracture Faces

Sampling. After each germination period, 10 root tips were fractured and five were taken randomly for quantitative study. Thirty fracture faces of the plasmalemma and 30 fracture faces of the nuclear membranes were analyzed in embryos germinated for 12 hr and more. In embryos germinated for 4 hr, 20 plasmalemma and 10 nuclear membrane fracture faces were analyzed. In ungerminated embryos the quantitative study was restricted to 20 fracture faces of the plasmalemma.

Diameter and Density of Nucleopores and Intramembranous Particles

Determinations of diameters and density of particles of the fracture faces of plasmalemma and nuclear membranes were performed on micrograph prints (× 60 000) with an apparatus that consisted of a stereomicroscope fitted with a calibrated ocular micrometer (Wild Censor Heerburgg M5). This allowed us to obtain a magnification of × 360 000.

The diameter of each particle was determined by measuring the widest part of the shadow at right angles to the direction of the shadow. This direction was indicated on each micrograph by an encircled arrow. Diameters of the nucleopores and of their constituent particles were measured in the same way.

To determine the numerical density of membrane particles, a test sytem composed of 999 squares of 36 mm^2 was superimposed on the micrographs and the particles were counted and measured inside every fourth square of the grid. Statistical analysis (variance) showed that this sampling procedure was adequate to determine the density of particles of the complete exposed fracture faces. The numerical density is expressed by the number of particles per square micrometer of freeze-fractured area.

For calculation of pore density, exposed fracture faces of the nuclear envelope were outlined and their areas determined by planimetry. The pores were then counted in these delimited areas. For nuclear-surface determinations, cross sections 1 μm thick were cut in root tips of embryos fixed in glutaraldehyde and OsO_4 and embedded in Epon (Crèvecoeur *et al.*, 1976). In each experimental series, 500 nuclei were randomly photographed with the light microscope and their diameters measured by means of an image analysis system (Leitz ASM).

The nuclear surface area was then calculated considering the nucleus as a sphere. The number of pores was expressed per square micrometer of nuclear surface.

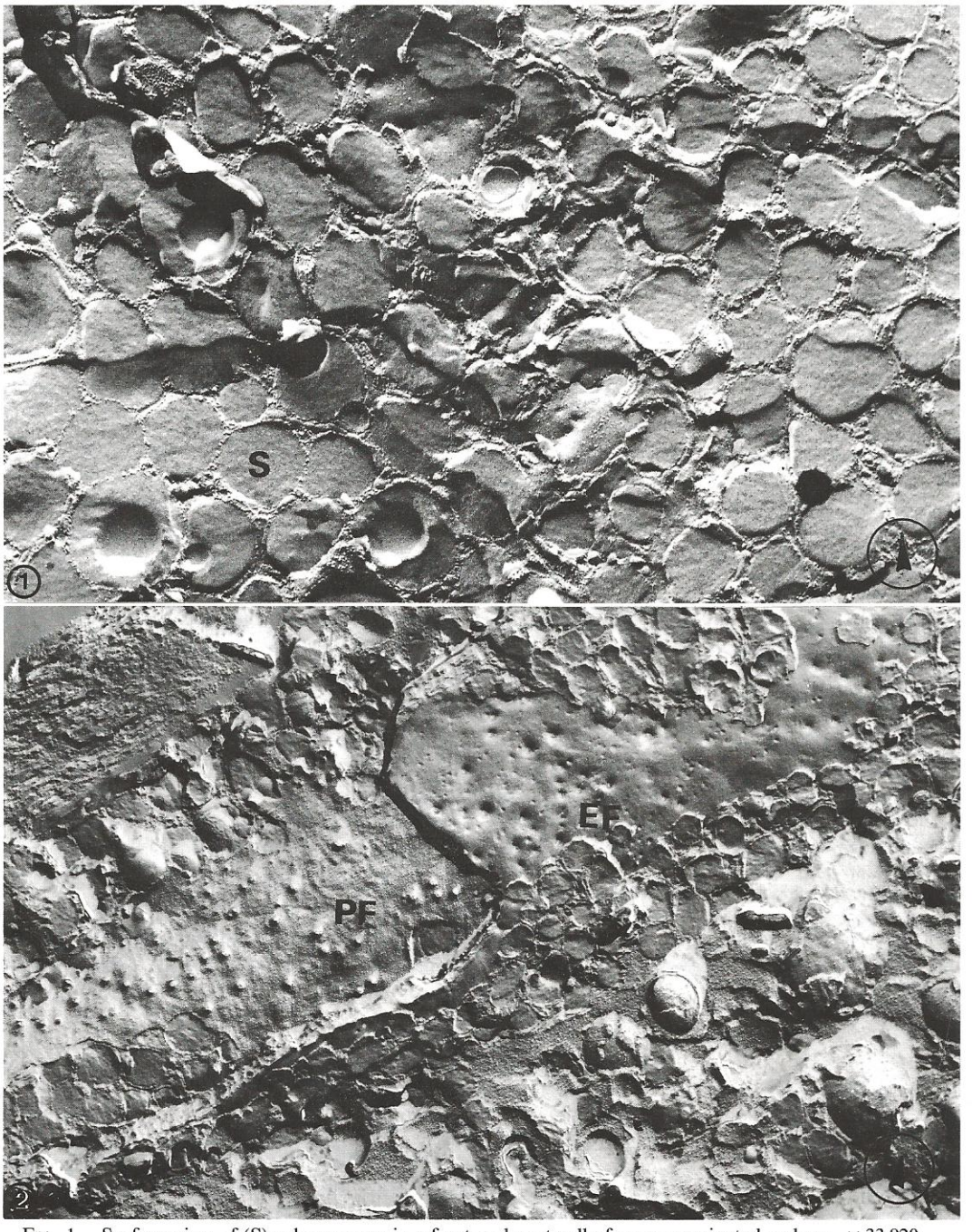


FIG. 1. Surface view of (S) spherosomes in a fractured root cell of an ungerminated embryo. × 33 920.

FIG. 2. Appearance of EF and PF fracture faces of the plasmalemma in a cell of an ungerminated embryo. × 16 900.

Statistical Analysis

Quantitative results relative to nucleopores and membranous particles were analyzed by means of the Mann-Whitney test and by variance analysis (Dagnelie, 1975).

Nomenclature of Membrane Fracture Faces

We designate the fracture faces of the plasmalemma and the nuclear membranes according to nomenclature proposed by Branton *et al.* (1975).

The plasmalemma fracture face nearest to the cell wall is termed EF, while that closest to the cytoplasm is termed PF.

The PF faces of the inner and outer nuclear membranes designate the half membranes closest to the nucleoplasm and to the cytoplasm, respectively. The EF faces of the two nuclear membranes correspond to the half membranes closest to the perinuclear space. Nuclear surfaces exposed by fracturing appeared generally either convex or concave, depending on the path of the fracture with respect to the nucleus (Severs and Jordan, 1975). When the fracture is directed upward and over the nucleus, a convex fracture face is produced that exposes EF faces of the outer nuclear membrane and PF faces of the inner membrane (Fig. 5). On the contrary, a fracture directed downward and under the nucleus results in a concave fracture on which we can see EF faces of the inner membrane and PF faces of the outer (Fig. 6).

Microdensitometry

Ultrathin cross sections were cut in root tips of embryos prepared following a conventional technique and stained with saturated aqueous uranyl acetate and Reynold's lead citrate (Crèvecoeur *et al.*, 1976). Thickness of the plasmalemma was measured from electron microscope negatives (× 20 000) using a double beam recording microdensitometer (Joyce Loebl 3CS). Densitometric tracings were recorded on the chart paper at 50 times expansion. The baseline of the densitometric tracing was given by the density of the cell wall. The thickness of the plasmalemma layers was estimated from the width at the half maximal value for a microdensitometer peak.

RESULTS

Plasmalemma

In quiescent embryos, as in embryos germinated for 4 hr, the fractures at the periphery of the cells frequently exposed the monolayer of spherosomes lying close to the plasmalemma (Crèvecoeur *et al.*, 1976) (Fig. 1). Some fractures, however, were through the plasmalemma and exposed a small area of its two inner faces that appeared covered with randomly distributed particles about 100 Å in diameter (Fig. 2).

In root cells of embryos germinated more than 4 hr, inner faces of the plasmalemma were more frequently seen on the replicas and appeared as larger surfaces (Figs. 3 and 4). The average diameter of particles of the two inner faces did not change during the first 72 hr of germination (Table I). From comparison of Figs. 3 and 4 and from data in Table I, it is clear that PF fracture faces are much richer in particles than EF fracture faces.

An increase of particle density was observed on EF fracture faces during the first 24 hr of germination (Table I). Statistical analysis has shown that this increase becomes significant at the 0.01 level of probability between 12 and 24 hr. On the con-

TABLE I

MEAN DIAMETERS AND NUMERICAL DENSITY OF PARTICLES OF INNER FACES OF THE PLASMALEMMA DURING GERMINATION[a]

Germination time (hr)	Average diameter (Å) of particles on		Mean numbers of particles per square micrometer on	
	EF fracture faces	PF fracture faces	EF fracture faces	PF fracture faces
0	106 ± 3.20	105 ± 4.15	208 ± 32	1120 ± 118
4	105 ± 3.25	104 ± 2.75	220 ± 40	1050 ± 95
12	107 ± 2.01	108 ± 1.17	240 ± 12	1170 ± 95
24	108 ± 0.96	106 ± 0.98	410 ± 48	1380 ± 83
72	107 ± 1.69	105 ± 0.84	420 ± 40	1370 ± 66

[a] ± Standard error of the mean.

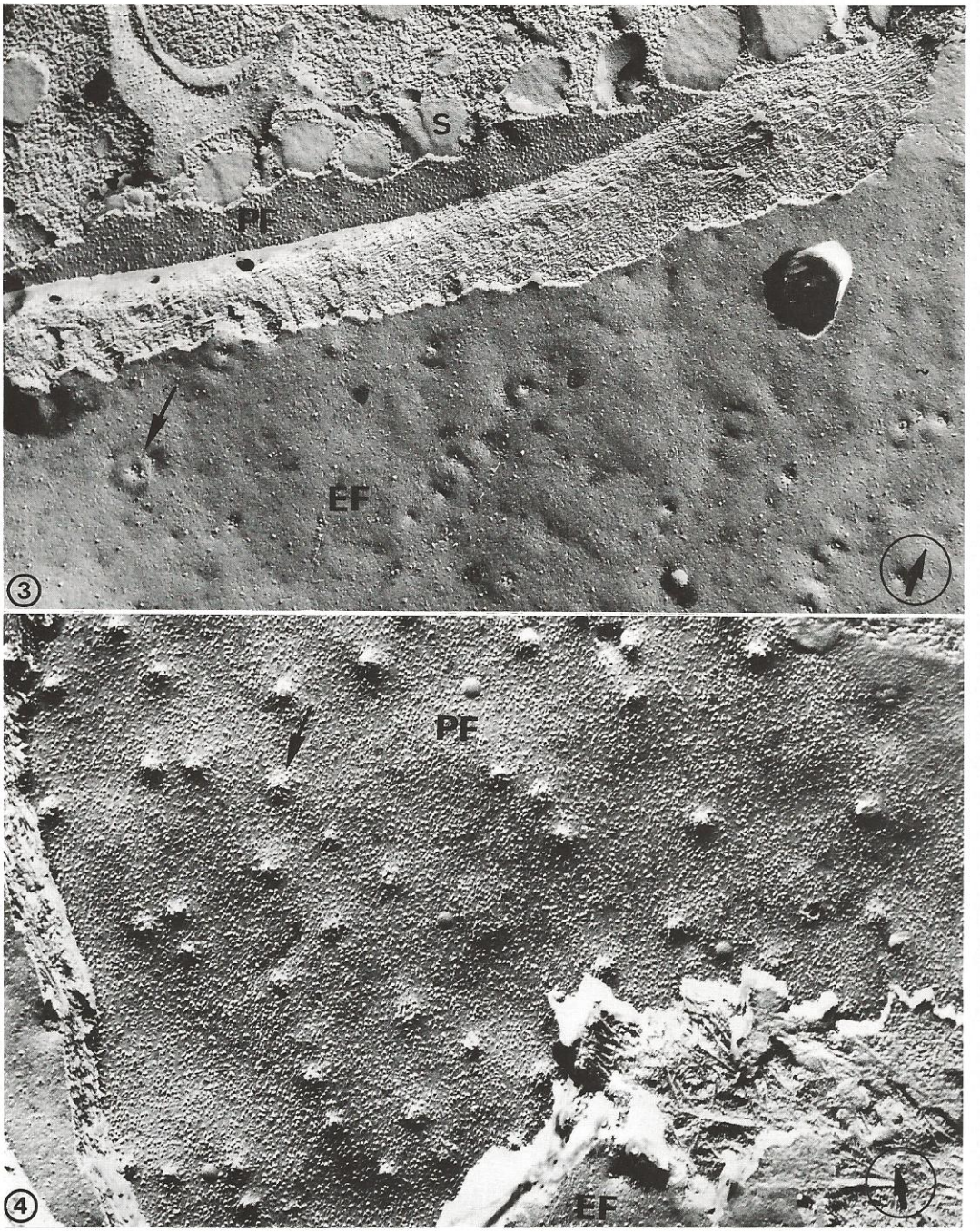


FIG. 3, 4. Portions of the two inner faces of the plasmalemma at the 24th hr of germination. Arrows indicate prints of plasmodesmata. Note the presence of a few (S) spherosomes in Fig. 3. × 46 000.

TABLE II

DEVELOPMENT OF PLASMALEMMA THICKNESS OF EMBRYONIC ROOT CELLS DURING GERMINATION (± SE)

Germination time (hr)	Thickness (Å) of plasmalemma and its constitutive layers			
	Total thickness	Inner dense layer	Light-central layer	Outer dense layer
0	100 ± 3.70	37 ± 0.99	28 ± 0.35	35 ± 0.82
24	115 ± 4.10	39 ± 1.25	38 ± 1.15	38 ± 1.10
72	114 ± 2.31	39 ± 1.35	37 ± 1.85	38 ± 0.61

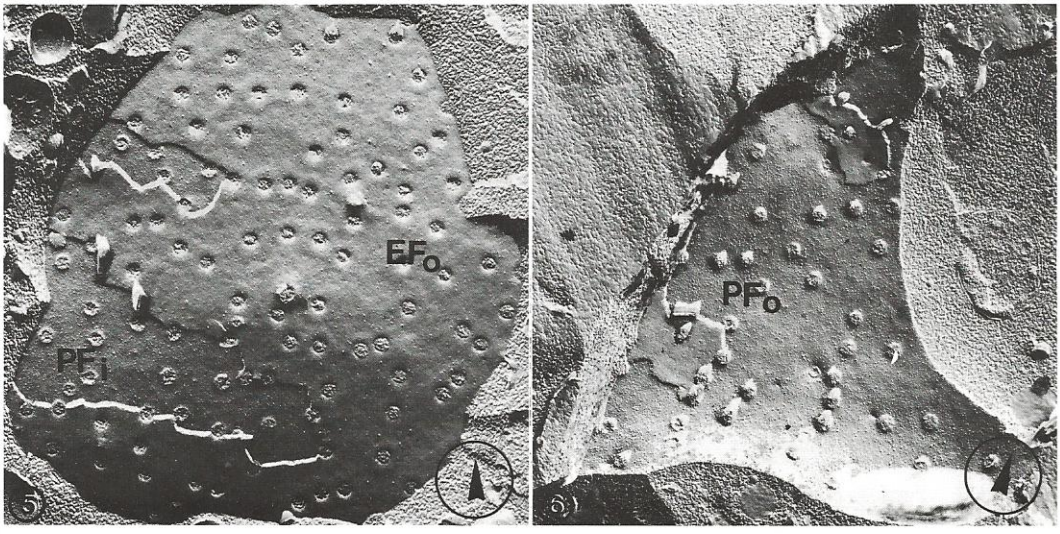
trary, the density of particles associated with PF fracture faces remained unchanged during this period.

At all times during germination, the two inner faces of the plasmalemma also showed prints of plasmodesmata, which appear as protuberances on PF faces (Fig. 4) and as holes on EF faces (Fig. 3).

The thickness of the plasmalemma of 24-hr embryos showed an increase of about 15 Å over that of ungerminated embryos (Table II). This increase was significant at $P < 0.01$ and was mainly due to the increase of thickness of the central layer. Thickness of the plasmalemma did not change between 24 and 72 hr of germination.

Nuclear Envelope

The fracture faces of the two nuclear membranes appeared almost entirely devoid of particles in cells of ungerminated embryos and of embryos germinated for 4 hr (Fig. 7). By the 24th hr of germination numerous particles were found on both faces but it is apparent that many more particles are found on the PF than on the EF fracture faces (compare Figs. 5 and 6, 9 and 10; see also data in Table III). The density of particles on EF and PF fracture faces



FIGS. 5, 6. Fractured root nuclei after 24 hr of germination.

FIG. 5. Convex fracture which reveals the (PF_i) PF face of the inner membrane and the (EF_o) EF face of the outer membrane. × 2000.

FIG. 6. Concave fracture which exposes the (PF_o) PF face of the outer membrane and the (EF_i) EF face of the inner membrane. × 20 000.

TABLE III

DEVELOPMENT OF THE DENSITY OF PARTICLES ON EF AND PF FRACTURE FACES OF THE TWO NUCLEAR MEMBRANES DURING EARLY GERMINATION (± SE)

Germination time (hr)	Density of particles (mean number per square micrometer)			
	Inner nuclear membrane		Outer nuclear membrane	
	EF fracture faces	PF fracture faces	EF fracture faces	PF fracture faces
4	4 ± 2	19 ± 9	6 ± 3	26 ± 11
12	64 ± 11	444 ± 46	76 ± 5	419 ± 37
24	335 ± 40	1151 ± 123	249 ± 62	1344 ± 102
72	401 ± 40	1201 ± 108	272 ± 27	1500 ± 83

increased dramatically within the first 24 hr of germination and then remained constant until the 72nd hr.

Throughout germination, the nucleopores appeared as cylindrical projections studded with particles about 100 Å in diameter on the PF faces of the two nuclear membranes (Figs. 5, 6, and 10). On the EF faces, they appeared as cylindrical holes containing particles or as slightly raised domes with particles (Figs. 5–9). At the 4th hr of germination fibrillarlike elements are seen in some nucleopores in addition to the particles (Fig. 7). They are not observed in the nucleopores of embryos germinated for longer periods. On the other hand, from comparison of Figs. 7 and 8 with Figs. 10 and 9, it is clearly apparent that the number of nucleopore particles increases during early germination. After 24 and 72 hr of germination it is difficult to count the particles.

Quantitative results relative to the nucleopores are summarized in Table IV. An increase of the mean pore diameter (significant at $P < 0.05$) was noted between 4 and 12 hr of germination. The average number of pores per square micrometer did not vary during the first 24 hr of germination. It then increased significantly until the 72th hr (Table IV). When expressed per nucleus, the average number of pores showed a similar evolution (Table IV).

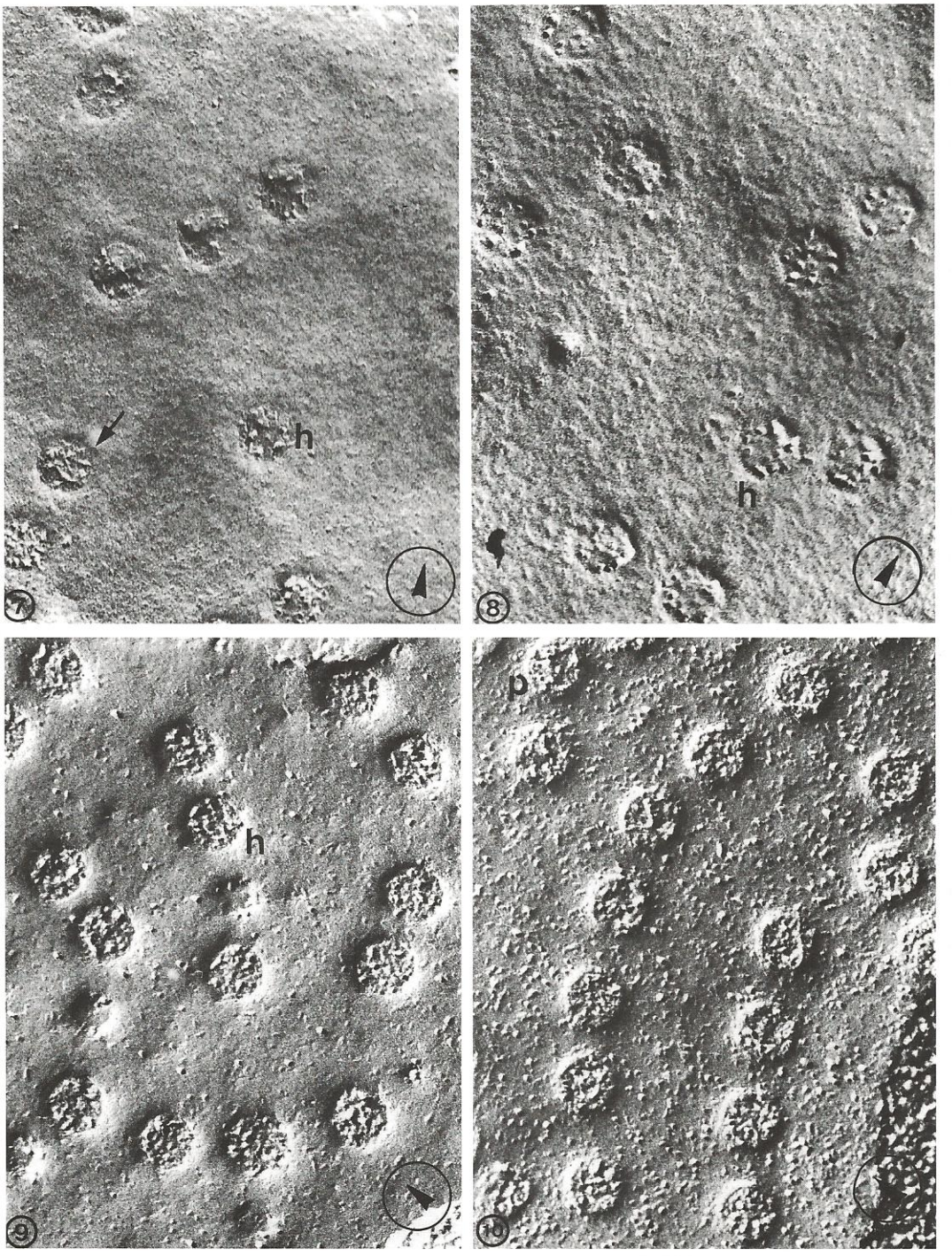
DISCUSSION

By use of the freeze-fracture technique, ultrastructural changes of the inner faces of the plasmalemma and the nuclear membranes have been revealed in root cells of maize embryo during early germination from the dry quiescent stage to 72 hr of germination.

Plasmalemma

In all experimental series, the two inner faces of the plasmalemma of maize root cells appear studded with particles about 100 Å in diameter, similar to those described in root cells of other embryos (Branton and Moor, 1964; Northcote and Lewis, 1968; Fineran, 1975). As in most of the freeze-fractured membranes, the density of particles in the plasmalemma is higher on the cytoplasmic side (PF) than on the external side (EF). Different interpretations have been proposed for this asymmetry and it is generally believed that most of the particles remain attached to the PF faces as a result of their deep penetration in this inside half of the bilayer membrane (for details see Bullivant, 1973).

No similar increase has been reported until now during germination. The only freeze-etching study that we are aware of on plasmalemma of ungerminated and imbibed seeds is the study of Buttrose (1971). He reports that the plasmalemma of aleurone cells of *Hordeum vulgare* appears typical and that it does not change during germination. No quantitative data are given, however. An increase in density of intramembrane particles has been reported in other tissues and related to increased mem-



FIGS. 7–10. Appearance of the nucleopores of freeze-fractured nuclei of germinated embryos. The EF face of the outer nuclear membrane is shown after 4 hr (Fig. 7), 12 hr (Fig. 8), and 72 hr (Fig. 9) of germination and the PF face of this membrane of a 72-hr embryo is presented in Fig. 10. Cylindrical hole (h)-type pores are seen on the EF faces and cylindrical projection (p)-type pores on the PF face (in Fig. 10). The arrow in Fig. 7 indicates the presence of fibrillarlike elements. × 92 400.

TABLE IV

QUANTITATIVE DATA RELATIVE TO NUCLEOPORES OF MAIZE DURING GERMINATION (± SE)

Germination time (hr)	Pore diameter (Å)	Mean number of pores/μm^2	Nuclear surface (μm^2)	Mean number of pores/nucleus
4	940	13 ± 1.02	401.15 ± 10.98	5.215
12	1022	12 ± 1.8	383.58 ± 12.32	4.603
24	1108	12 ± 1.6	430.78 ± 13.20	5.169
48	1020	16 ± 1.6	410.42 ± 10.86	6.567
72	1050	18 ± 3.2	427.10 ± 7.54	7.688

brane activities (Ojakian and Satir, 1974; Humbert *et al.*, 1975; Brown and Ilic, 1979).

Our observations of the plasmalemma during the first 24 hr of germination have also revealed an increase in thickness of its central layer, which is mainly composed of phospholipids (Staehelin and Probine, 1970). A similar increase has been reported for artificial lipidic membranes when fatty acid chain lengths increase from C14 to C22 (Haydon, 1975). During germination of *Pisum sativum,* fatty acids C16 to C18 long are synthesized first, followed by fatty acids from C20 to C26, which are mainly associated with various membranes (Harwood and Stumpf, 1970). These observations indicate that an increase of plasmalemma thickness during early germination could be due to changes in its lipidic composition.

Nuclear Envelope

The inner faces of the two nuclear membranes revealed 100-Å particles similar to those noted on fracture faces of the plasmalemma. Such particles are not uncommon in freeze-fracture studies of plant-cell nuclei (Branton and Moor, 1964; Northcote and Lewis, 1968; Fineran, 1975; Severs and Jordan, 1975).

A dramatic increase in particle density on the two fracture faces of both nuclear membranes is observed during the first 24 hr of germination. No similar account has been found in the literature.

Nucleopore diameters and densities that we found are comparable to those reported for different plant tissues (Thair and Wardrop, 1971; Maul, 1977). The density of pores undergoes a significant increase between 24 and 72 hr of germination and similar increases are reported during activation of dormant carrot root cells (Jordan and Chapman, 1973), during early stages of germination in cotyledons of *Cucurbita maxima* (Lott and Vollmer, 1975), and in other tissues (see Franke, 1974; Maul, 1977). Nuclear-pore function has been related to transcription (Leaver and Key, 1967; Jordan and Chapman, 1973; Lafountain and Lafountain, 1973; Zerban and Werz, 1975) and to DNA duplication (Scott *et al.*, 1971; Lott et Vollmer, 1975; Maul, 1977; Schel *et al.*, 1978). We show here that the increase of nucleopore density starts later than resumption of transcription (Van de Walle *et al.*, 1976). Consequently a relationship between these two phenomena must be excluded. On the contrary, the DNA duplication starts between the first and the second day of germination (Deltour and Jacqmard, 1974; Crèvecoeur, unpublished) and the rise in nucleopore density occurs concomitantly. Hence as far as timing is concerned, our results argue more for a DNA duplication function of the nucleopores.

The variation described in the appearance of the nucleopores between PF and EF faces of the nuclear membranes is characteristic of fractured plant-cell nuclei and can be explained in terms of the fracture path through the nucleopores (Severs and Jordan, 1975). We show here that the fine structure of the nucleopores changes during

early germination. It is noteworthy that this change is parallel to the increase of RNA synthesis and RNA nucleocytoplasmic translocation previously reported on the same material (Van de Walle *et al.*, 1976; Dommès and Van de Walle, 1980). This observation argues for the concept that the nucleopores are involved in RNA transport and that elements of the pore complexes are in part morphological expression of such a migration (Franke and Scheer, 1970; Maul, 1977).

The results of the present study indicate that the reactivating nucleus of the early germinating maize embryo is very suitable to investigation of relations between structure and function of the nuclei, i.e., transcription and duplication. Moreover, they emphasize that the nuclear envelope is actively involved in this reactivation.

On the other hand, we think that attention must be payed to the extremely low density of particles in membranes, mainly nuclear membranes, of quiescent embryos. This is probably a characteristic of the quiescent cell and reflects their very low metabolic status.

Another result which is important to underline is the enrichment of two cellular membranes in particles (IMPS). What is the significance of such an enrichment? This poses the question of the chemical nature of membrane particles exposed by the fracture process. There are strong indications that IMPS are proteins or aggregates of proteins intercalated into the membrane lipidic bilayer, although the possibility that the particles are lipids cannot be ruled out (Staehelin and Probine, 1970; Tillack and Marchesi, 1970; Hong and Hubbell, 1972; Ruben *et al.*, 1976; Verkleij and Ververgaert, 1978). It has been suggested that they represent, more precisely, enzymes or enzyme complexes and a correlation has been proposed between rise in density of particles and higher activities of the plasmalemma (Staehelin, 1968; Roland, 1973; Chailley, 1979). Activity of some enzymes has been shown to appear at the membrane level during germination in embryos of *Allium* (Colson, 1979) and of *Zea* (Deltour *et al.*, 1981). This lead us to suggest that the increase in density of IMPS that we report could reflect the insertion of new plasmalemma and nuclear membrane-associated enzymes. Further experiments are underway to confirm this suggestion.

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