Application of Freeze Etching Technique for Investigating Cell Wall Organization of Parenchyma Cells in Higher Plants

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Abstract——Freeze etching technique was applied for investigating cell wall organization of parenchyma cells in the cortex. This technique gave highly oriented microfibrils in any lamellae and did very good insight into three dimensional relationship among cytoplasm, plasmalemma and cell wall. The technique can also give information of the orientation of microfibrils inherent to respective lamella which makes wall by piling up in a complex manner. Hence, freeze etching technique seems more reliable for the study of natural states of cell wall organization than the classical direct shadow casting technique and more recent replicating, followed by shadow casting techniques.

Introduction

In the investigation on cell wall organization of parenchyma cells of primary tissues, the following series of processes had been adopted: (1) blending; the fibers are separated mechanically by means of a small electric blender, (2) maceration; the fragmented coleoptiles are macerated by alternate treatments with dilute alkali and dilute acid, (3) mounting on sheet meshes; after washing of the macerated fibers, the fiber suspensions are dropped on collodioncoated grid meshes and air-dried, (4) shadowing; materials on the grid meshes are shadowed by any one of Cr, Pt or U.

These are a series of techniques to observe directly the shadowed materials by means of electron microscope. Thereafter, other method[1] was developed, that is, delignified materials were embedded in methacrylate resin, and then ultrathin sections were obtained, deembedded and shadowed by Pd-Au alloys. Sterling[2] demonstrated the crossed fibrillar structure in developing fiber of *Asparagus* by using an alternative method; parafin-embedded materials of 2~5 μm sections were deembedded, and their replicas were prepared by shadowing and carbon-backing. These are a modified form of direct carbon replica technique.

Côté[3] pointed out that direct carbon replica technique was simple, nondistorting, reproducible and highly reliable one in representing fine detail. On the other hand, the preparation of tissue in natural condition has been improved. Particularly, attempts to use freeze drying methods, solvent exchange drying methods and critical point drying methods have been made with varing results by Thomas[4,5], Thomas

and Nicholas[6], Imamura and Harada[7], and Sachs and Kinney[8] in order to observe the structure of pit membranes in green conditions. Norberg[9] presented a new method for investigating wet wood fiber surfaces.

Freeze etching which is the more improved technique has been widely applied to observe surface and three-dimensional structures of the organelles of higher plant cells[10~12]. This technique, however, has not been used as a mean to investigate the organization of lamellae which compose the cell wall of higher plants, except a slight description by Chafe and Wardrop[13], and Puritch and Johnson[14].

The present paper compared the freeze etching techinique with freeze drying followed by replicating and shadow casting techniques and with the classical shadow casting techniques to clarify the advantages of the former in studing the organization of soft cell walls such as those of parenchyma cell.

Meterials and Methods

The following plants were used as experimental materials : *Pinus thunbergii* Parl., *Populus nigra* L. var. *italica* Koehne, *Morus bombycis* Koidz. and *Phaseolus vulgaris* var. *humilis* Alef.

Seeds of both *Pinus* and *Phaseolus* were grown in wet vermiculite in a dark room at 28°C. A few day's seedlings of *Pinus* after germination were used. Young shoots of poplar which elongated from the branch cutted in resting time in water in a dark room at 28°C were used. Besides, natural shoots of *Morus* grown early in the season were used.

Freeze Drying

Freeze drying followed by shadow casting techniques were attempted in this study for the comparison with freeze etching technique, since freeze drying technique seems to minimize the artifacts due to the shrinkage of the specimens among the previous preparating techniques.

Fresh materials were cut in small pieces, which were plasmolysed in 0.8~1.0 M sucrose solution. Sections of about 100 μm thickness which were prepared by freezing microtome were sandwitched between two slide glasses.

The sandwitched materials were put into test tube (ϕ 30 mm) which were cooled by mixture of sodium chloride and ice water. After completely freezed, the materials were maintained under reduced pressure. The cold-trap which was composed of dry ice and acetone was settled between a test tube and a rotary pump. Freeze dried materials were shadowed by Pt-Pd alloys and carbon-coated.

The dissolution of materials from the thin firms of replicas was successfully performed without the application of backing layer.

Freeze Etching

Materials were cut in a small pieces using razor blades and fixed in 2.5% glutaraldehyde solution (pH 7.2). After thoroughly washing, they were immersed in 50% glycerol solution.

Freeze etching apparatus used was that of the block type which was designed by Nishiura[15]. After the materials were put on a specimen holder, the whole block was frozen completely in a liquid nitrogen. Then, the whole block was transferred into a vacuum evaporator equipped with a cold trap. When the pressure of 1×10^{-5} Torr was obtained, the materials were fractured by knife settled in the block. If necessary, the materials were etched by heating them up to $-90°\sim-100°$C.

The dissolution of materials from the replicas obtained by freeze drying and freeze etching techniques was accomplished by the following processes: (1) replicas were immersed in acidified sodium hypochlorite solution, (2) cellulose and other polysaccharides were hydrolysed in 72% sulfuric acid, (3) undissolved debris in the above two processes were removed by ordinary household chlorine bleach. Clean replicas obtained were put on a grid meshes (200 mesh) and examined with JEM T6S electron microscope.

Results and Discussion

Great many contributions have been presented on the organization of primary walls[16~19]. They presented a remarkable consistent picture, that is, the inner layer of primary wall consisted of perpendicularly oriented microfibrils to the main cell axis, while the outer layer consisted of randomly or longitudinally oriented ones. The occurrence of such a structure has been uniformly interpreted by the multinet growth hypothesis.

In the present study, the structure of cortical parenchyma cell walls in the primary tissue of young shoots was investigated. Although it was not confirmed that this type of cell wall was being deposited during the phase of surface growth, parenchyma cells in the cortex were thought to have primary wall[20].

The previous replica techniques including freeze drying followed by shadow casting one were useful for the investigation of microfibrillar orientation of primary wall in a point where the oriented microfibrils in a large area are seen. The following disadvantages, however, were noted in these techniques.

(1) Wavy pattern of microfibrils are sometimes found (Fig. 1). Similar phenomena can be seen in the reports of Roelofsen and Houwink[21] (in Fig. 7), Wardrop[22] (in Plate 2), Setterfield and Bayley[23] (in Plate 1-Fig. 2), Moor[24] (in Fig. 26), and Imamura *et al.*[7] (in Fig. 5).

(2) Three dimensional relationship is not so clear between each lamella and

among cytoplasm, plasmalemma and cell wall.

(3) Randomly oriented microfibrils are found very often (Fig. 2), which may occur because of the displacement of microfibrils during the preparation of materials in each step of "blending" and "maceration".

(4) Bundled microfibrils are frequently observed (Fig. 3). Similar phenomena can be seen in the reports of Houwink and Roelofsen[25] (in Plate 23 b), Böhmer[26] (in Fig. 17 and 22), Wardrop[27] (in Plate 2-Fig. 1), Wardrop[22] (in Plate 2) and Imamura *et al.*[7] (in Fig. 2 and 6).

(5) Tissue shrinkage may occur in any replicating methods during drying pro cesses except freeze etching.

Contrary to the previous replica techniques, freeze etching technique has many advantages as follows :

(1) Fixation and glycerol impregnation are the only processes which may induce some artificial modifications of tissues in this technique. It is incredible that either of these processes may introduce some morphorogical changes in the arrangement of wall microfibrils.

(2) Highly oriented microfibrils are commonly seen in each lamella (Figs. 4, 5 and 6), while bundled or wavy microficrils are not found at all.

(3) Three dimensional relationship between respective lamella and among cytoplasm, plasmalemma and cell wall is clearly recognized as shown in Fig. 7.

(4) Orientation of microfibrils inherent to a lamella is clearly discernible even if lamellae are piled up in a complex manner as shown in Figs. 8 and 9.

(5) Polylamellate structure of parenchyma cell walls is easily seen (Fig. 10). Chafe and Wardrop[13] investigated the structure of epidermal cell walls of several species which shows polylamellate structure. The experimental materials in this study is thought to have a similar structure. In fact, epidermal cell walls of *Phaseolus* observed by freeze etching technique show a polylamellate structure in which all lamellae tend to be thinner near the cuticle (Fig. 11), as described by Chafe and Wardrop[13]. Because of the occurrence of a similar structure, it is strengthened that parenchyma walls have a polylamellate structure.

(6) It is posible to determine the width of microfibrils *in sttu*. Fig. 12 shows such microfibrils which do not suffer from any drastic chemical treatment or severe drying.

However, it is not so easy to obtain freeze etched replicas of plant tissue because of the greatest difficulties encountered in the final steps of releasing the replica from underlying tissue. The carbon-platinum replicas are extremely fragile and brittle and, therefore, can not be directly manipulated without danger of breakage at the boundary between cytoplasm and cell wall. Fracture plane must proceed through the internal

surface of the cell wall in order to observe the organization of cell wall, but rapidly elongating parenchyma cells possess quite thin walls so that the probability of fracturing through the walls is much less frequent. Hence, the freeze etching technique is quite time consuming in the application for investigating cell wall organization of plant tissues.

In spite of the shortcomings mentioned above, freeze etching technique greatly reduces the artifacts and is highly reliable in representing the orientation of microfibrils in natural condition than the classical shadow casting technique, and freeze drying followed by replicating and shadow casting techniques.

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Explanation of Figures

Abbreviations used:

CW = cell wall
Ep = epidermal cell
V = vacuole
$\widehat{PL}$ = convex plane of plasmalemma
PPF = primary pit field
Cy = cytoplasm
To = tonoplast
Pa = parenchyma cell
$\underset{\smile}{PL}$ = concave plane of plasmalemma
⇔ = direction of shadowing

Fig. 1. Freeze dried cell wall of *Pinus* parenchyma cell. Wavy pattern of microfibrils is shown. ×20,000.

Fig. 2. Freeze dried cell wall of *Phaseolus* parenchyma cell. Randomly oriented microfibrils are shown. ×27,000.

Fig. 3. Freeze dried cell wall of *Pinus* parenchyma cell. Bundled microfibrils are shown (arrows). ×21,000.

Fig. 4. Freeze fractured wall of *Pinus* parenchyma cell. Highly oriented microfibrils are shown. ×9,000.

Fig. 5. Freeze fractured wall of *Phaseolus* parenchyma cell. Highly oriented microfibrils are shown. ×10,000.

Fig. 6. Freeze etched wall of *Populus* parenchyma cell. Highly oriented microfibrils are shown. ×24,000.

Fig. 7. Freeze fractured wall of *Populus* parenchma cell. Three dimensional relation among cytoplasm, plasmalemma and cell wall is shown. ×21,000.

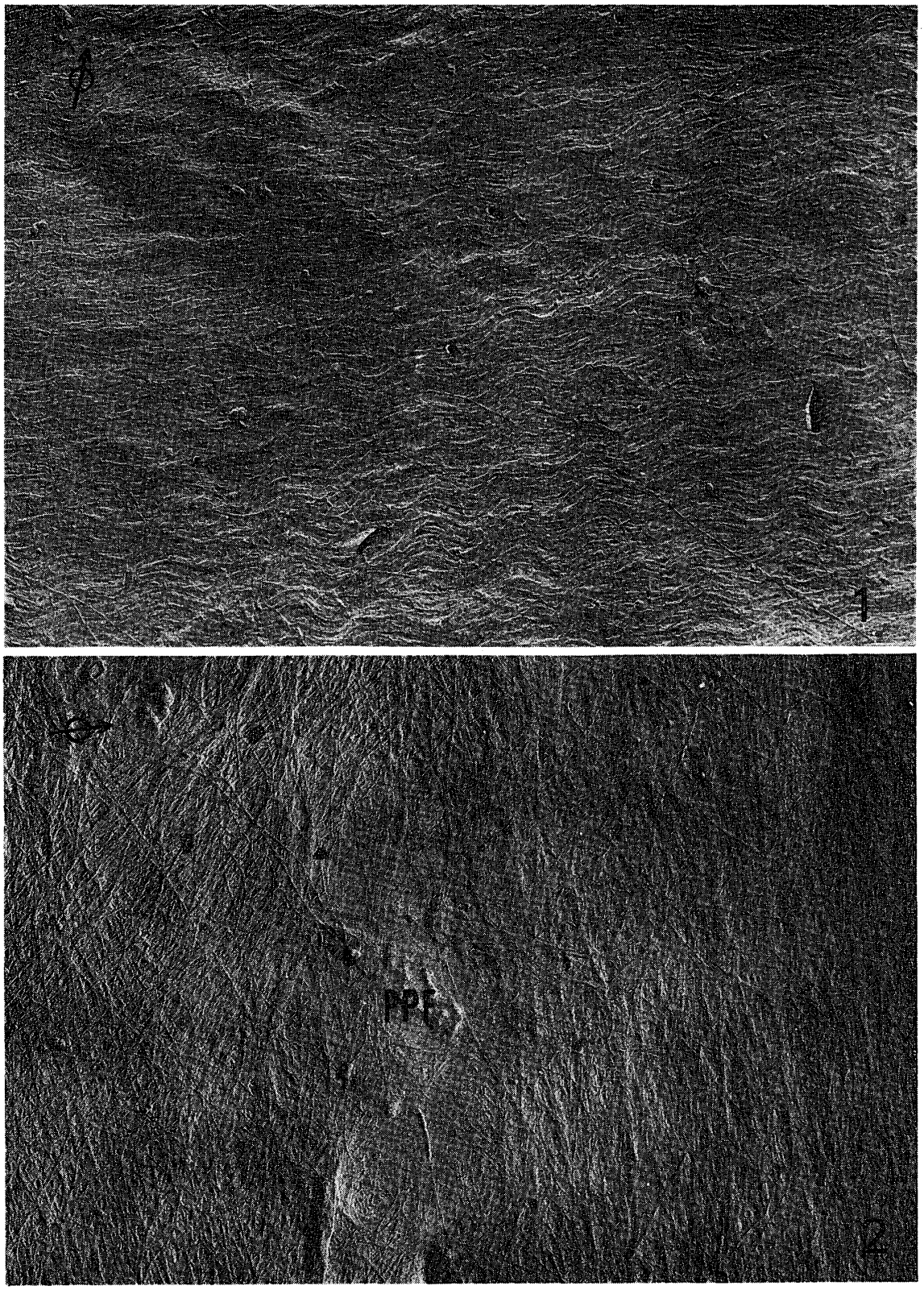
Fig. 8. Freeze etched wall of *Morus* parenchyma cell. Obliquely fractured wall is shown. Microfibrillar orientation inherent to each lamella is discernible even if lamellae are piled up in a complex manner. ×28,000.

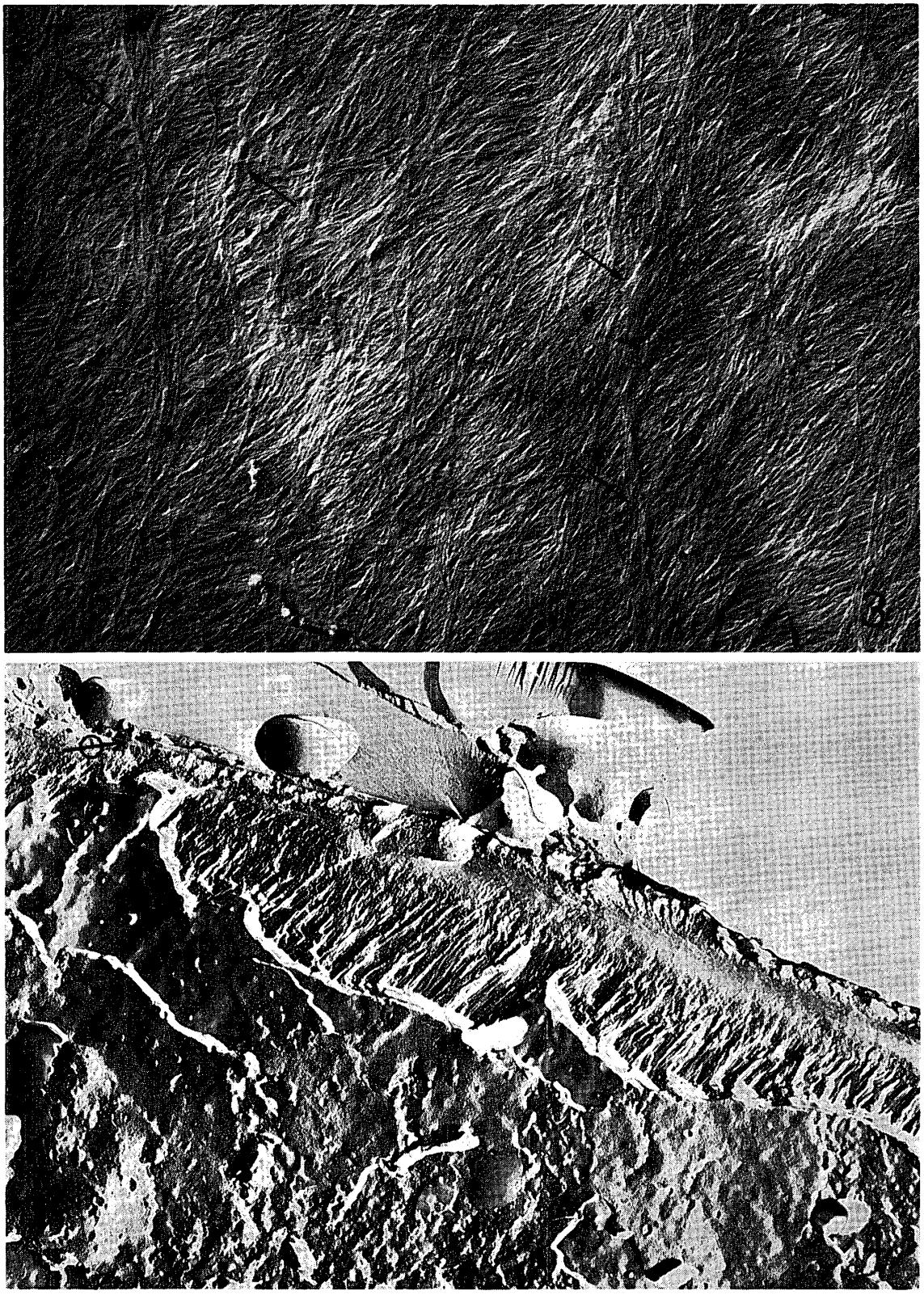
Fig. 9. Freeze fractured wall of *Populus* parenchyma cell. Several lamellae are piled up at complex manner. These lamellae are criss-crossed one another. ×21,000.

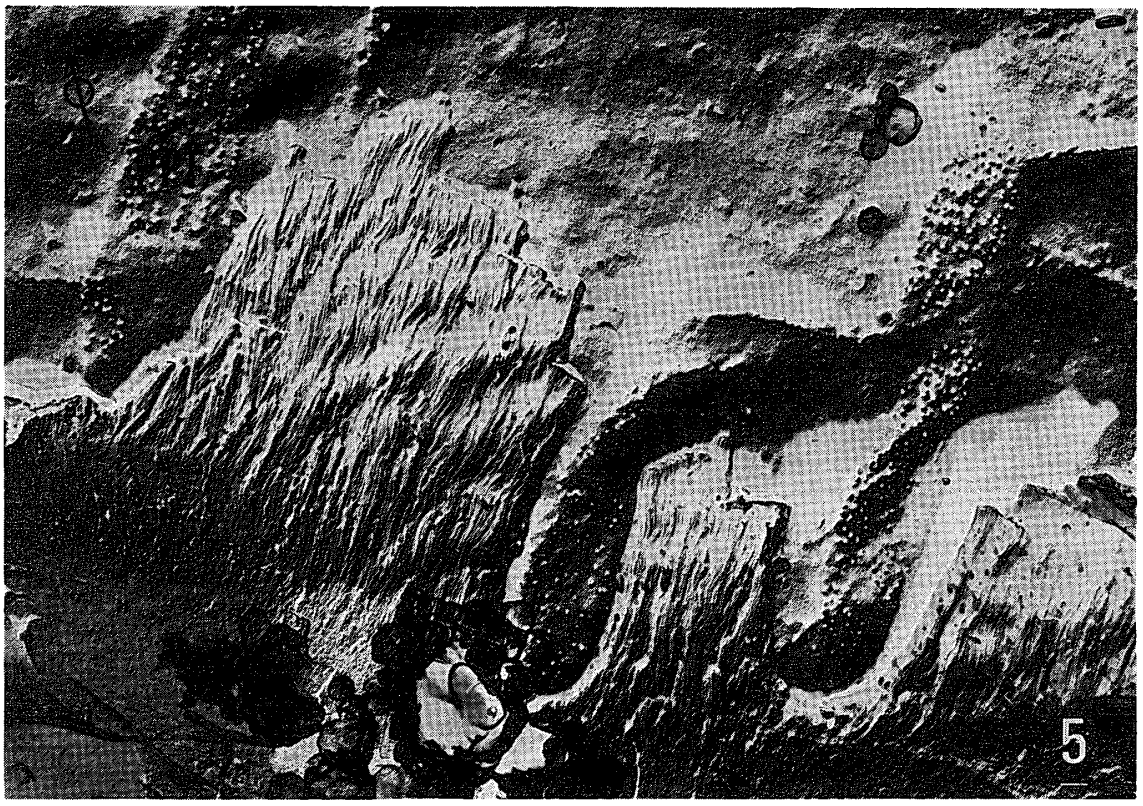
Fig. 10. Freeze etched wall of *Morus* parenchyma cell, which shows polylamellate structure. ×22,000.

Fig. 11. Freeze fractured wall of *Phaseolus* epidermal cell. Polylamellate structure in which lamellae tend to be thinner near the cuticle is clearly seen. ×9,000.

Fig. 12. Freeze etched wall of *Morus* parenchyma cell. It is possible to determine the width of microfibrils which do not suffer from any drastic chemical and mechanical treatments. ×28,000.



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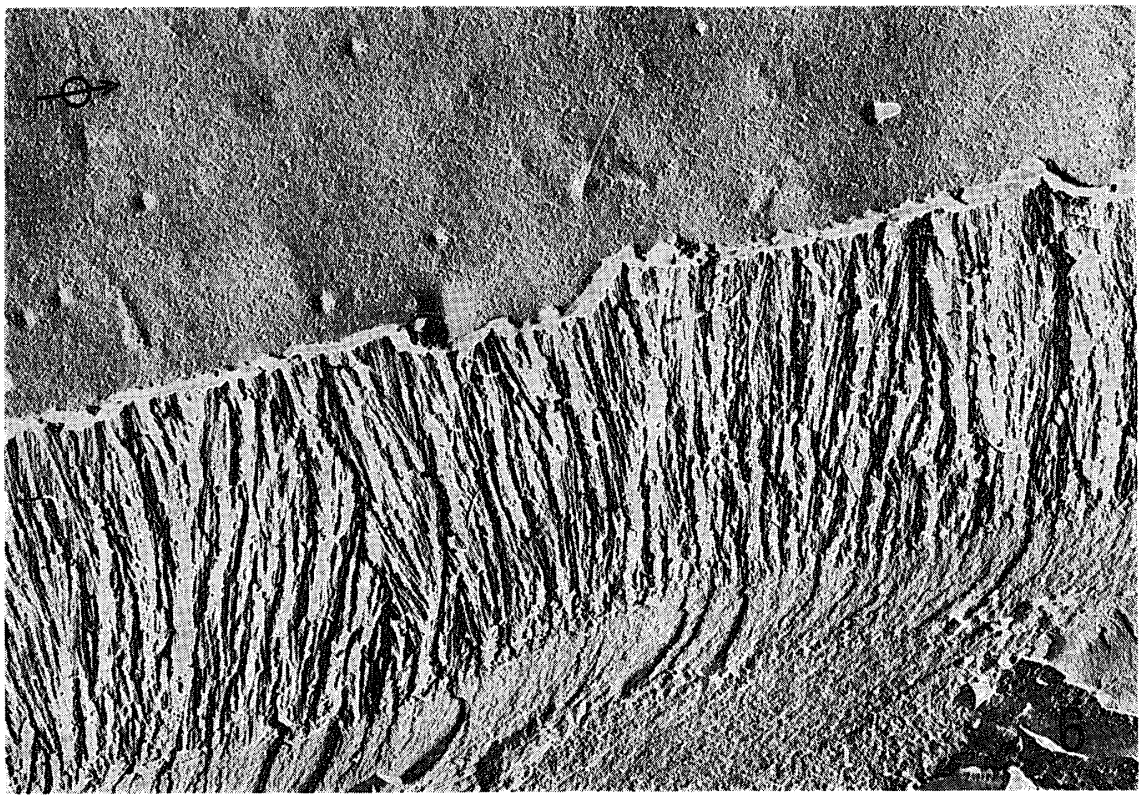

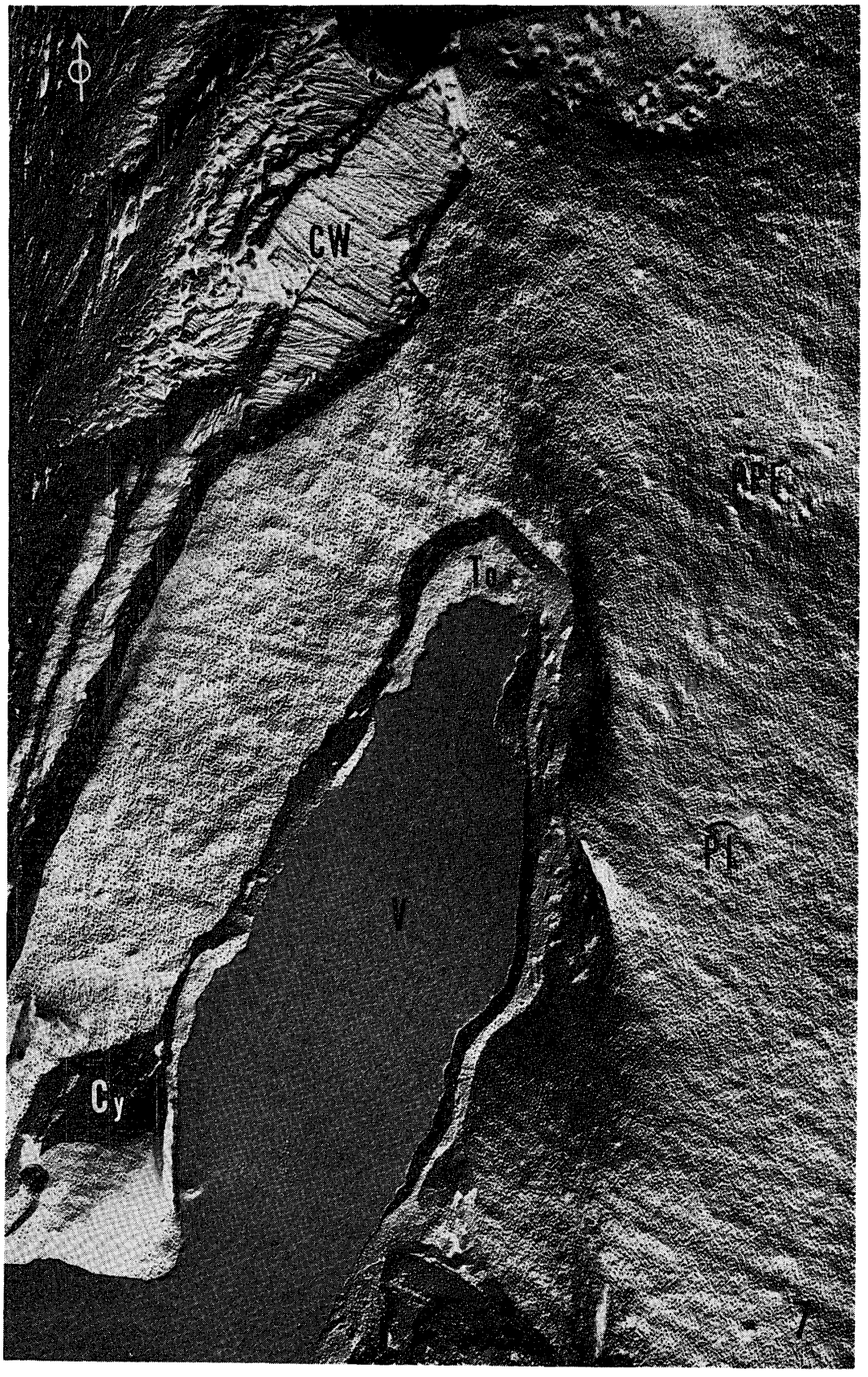
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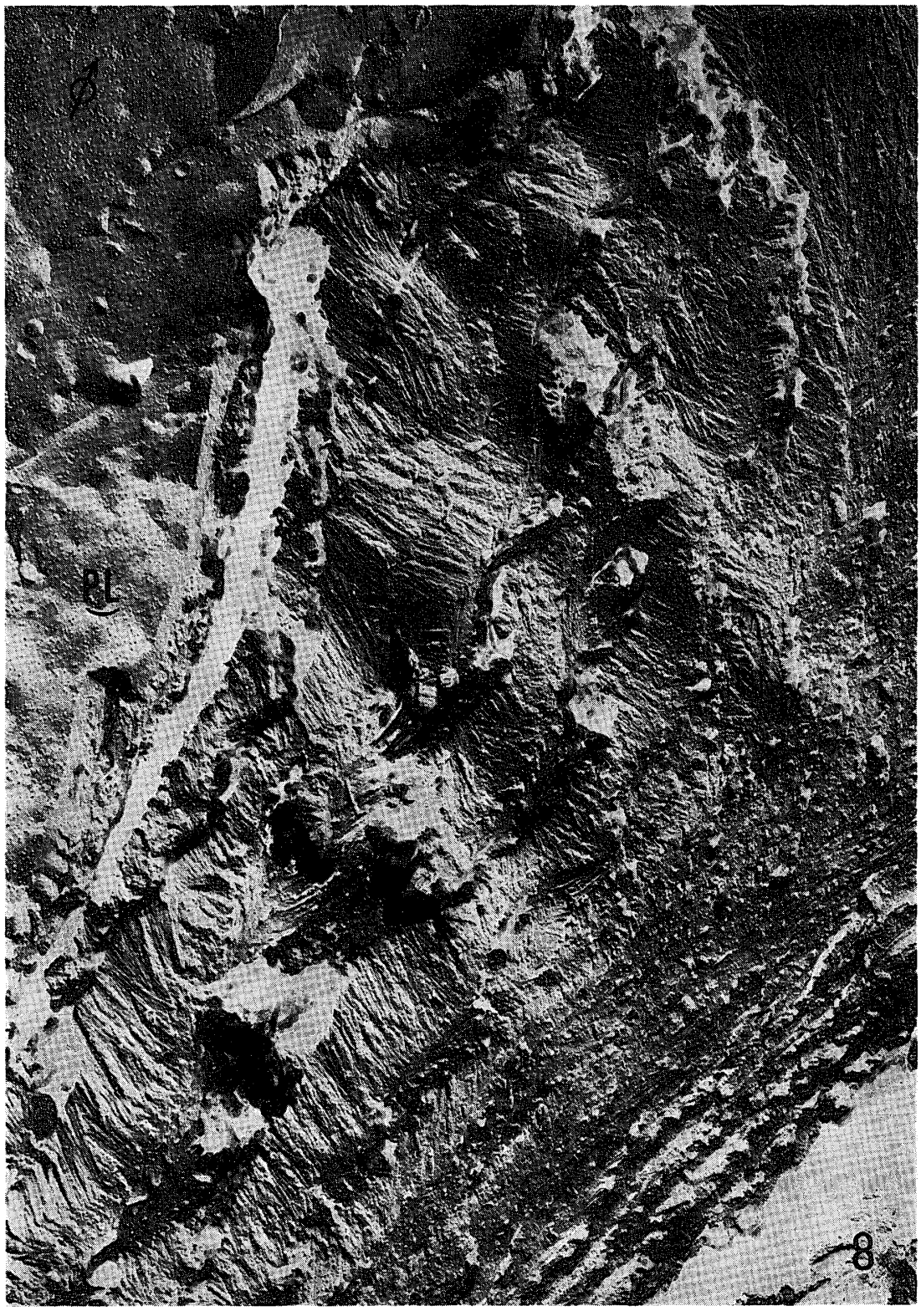

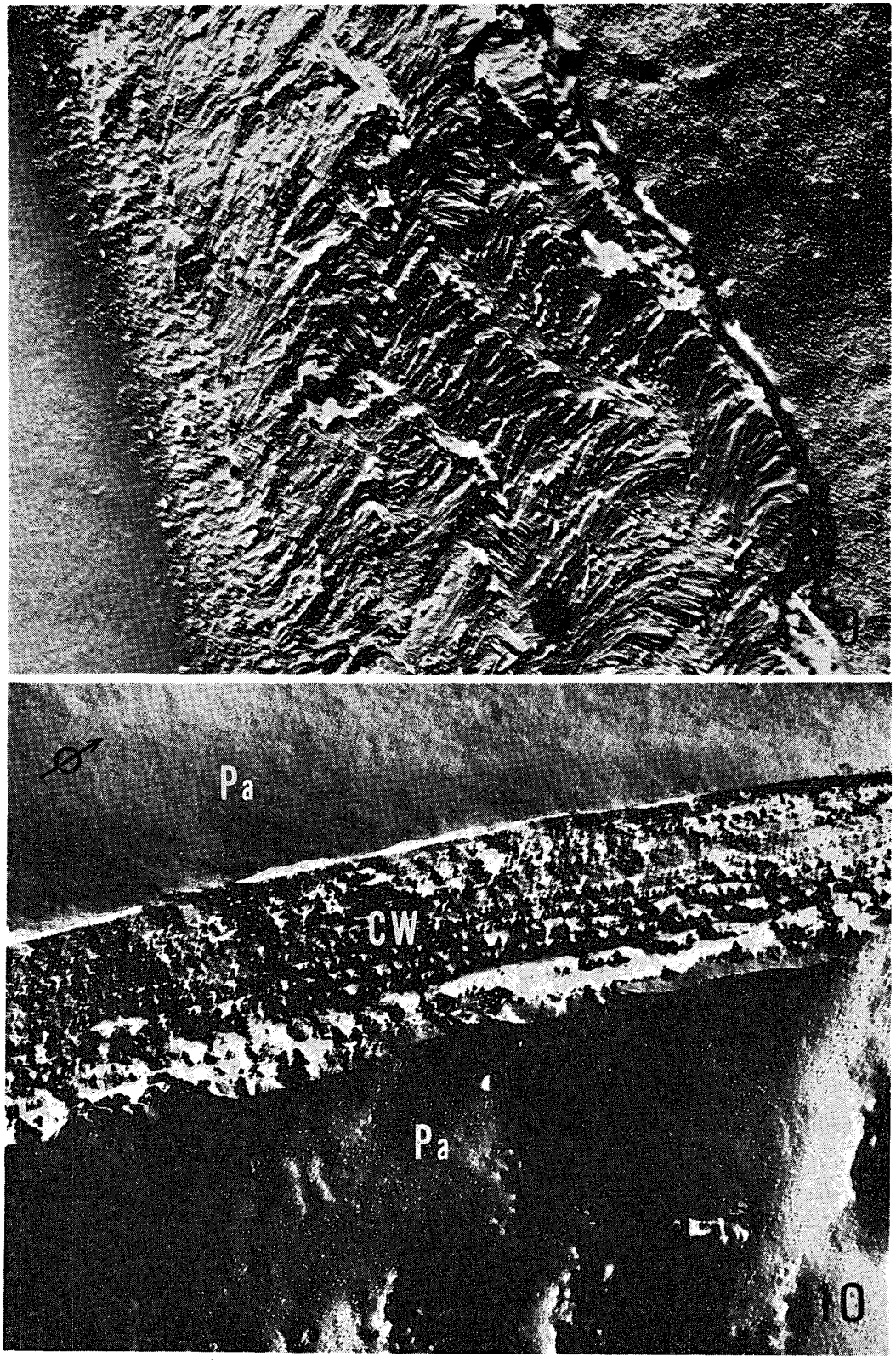
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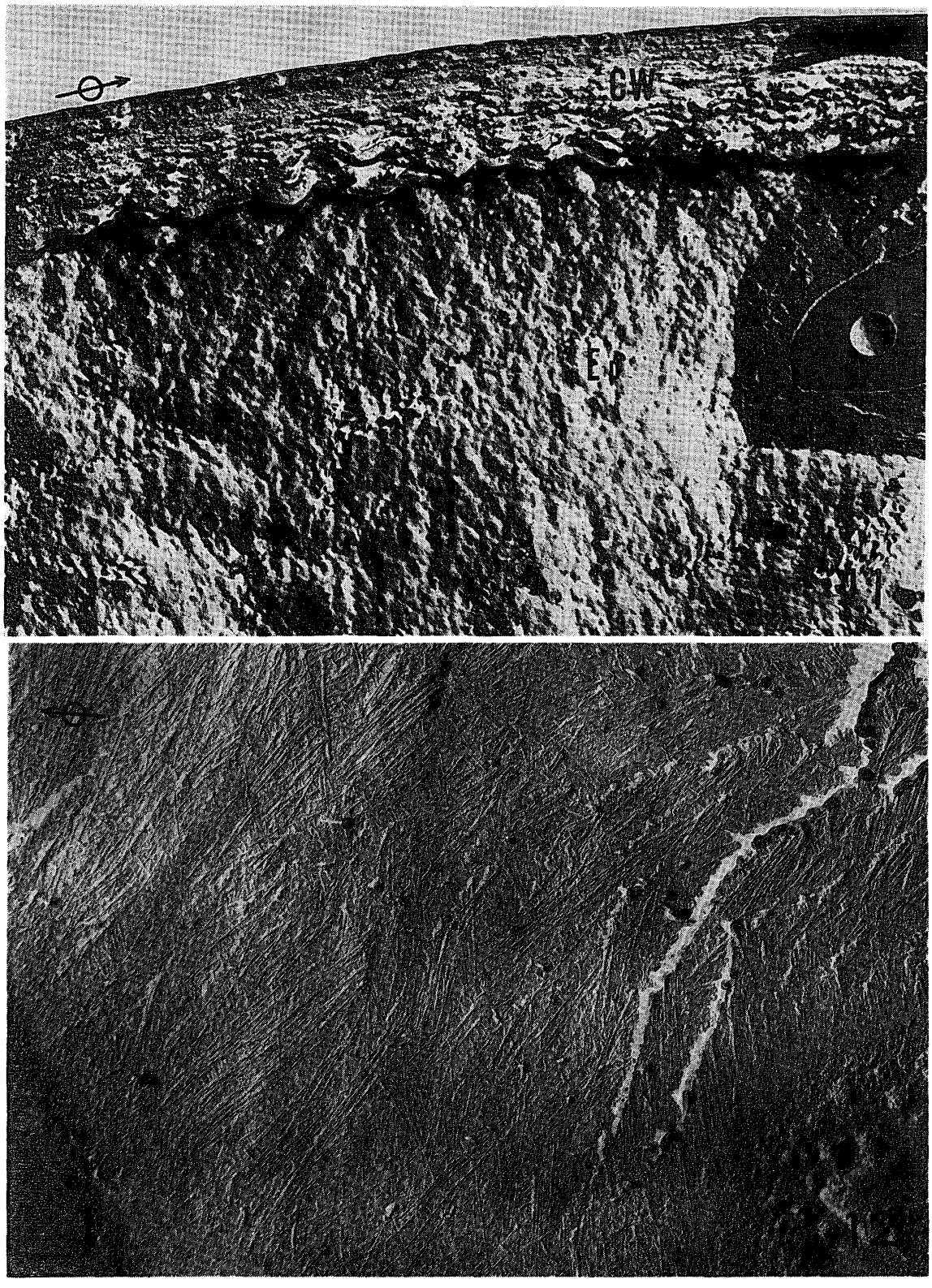
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