Review Article

# Studies on the Structure and Growth of Primary Walls of Woody Plants

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**Contents**



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## Introduction

The understanding of biological organization of cell walls of woody plants has been of great importance for both academic interests and practical utilization of wood. The cell wall is a highly functional entity which varies in composition and architecture, internally in accordance with cell to cell and cell to tissue interactions, and externally with environmental factors. The approach from the structural aspect of cell walls must eventually provide a basis for understanding the regulation of cell wall formation by genetic and environmental control factors.

All the plant cells pass through some or all of the following stages during their maturation: (1) cell division, (2) surface growth of primary wall, (3) secondary wall thickening and (4) lignification. The cell shape is determined during the second stage. From the standpoint of cell wall structure, it is quite interesting that almost all of plant cells expand until it reaches the final cell shape in spite of the rigidness of the cellulosic cell wall envelope. The cell wall in the stage of surface growth is generally recognized as a primary wall, but any clearcut understanding of its structure in the developmental process has not been established yet.

Since the wall is deposited outside the plasmalemma and new lamellae are continuously added during the life of a cell, changes in wall structure in a spatial sequence from the edge of the cell toward the inside express also a time sequence depicting the history of the cell maturation. Thus, it may be necessary to understand the cell wall structure from the synthetical standpoint.

The present study deals with the cell wall structure at various sequential stages of growth. The architectural details of a wall at any stage of the development of a cell must contain informations concerning the mechanism by which it has been elaborated, which can be obtained in no other way than the observation of sequential stages of cell growth. Besides, the present study purposed to clarify the significance of the freeze etching technique for investigating the cell wall structure such as of flexible and hydrated primary wall which has to be observed in the more intact condition.

Chapter 1 deals with the significance of the freeze etching technique in investigating the surface structure of plasmalemma and the orientation of microfibrils in relation to the structure and growth of primary wall. Materials used in this chapter are cortical parenchyma cells of poplar (*Populus nigra* L. var. *italica* Koehne). In Chapter 2, the changes of wall thickness and the orientation of microfibrils and microtubuls during cell elongation are first discussed. Then, the occurrence of crossed polylamellate structure during cell elongation is discussed. In Chapter 3, the behavior of the cortical parenchyma cell wall expanded radially by the application of growth inhibitors such as coumarin and colchicine is investigated with pine (*Pinus thunbergii*

Parl.) seedlings. Finally, the wall structure of suspension-cultured cells of *Rauwolfia serpentina* Benth and *Nicotiana tabacum* L. without polarity in cell growth is investigated in Chapter 4.

## 1. Significance of the freeze etching technique for the investigation of microfibrillar orientation

### 1.1 Early informations obtained by the electron microscopy

One who wants to know the cell wall organization of hydrated cells such as parenchyma cells, he must seek more reliable method to observe the orientation of microfibrils in the green condition close to their natural state.

In investigating the cell wall organization of parenchyma cells of primary tissues, the following series of processes were adopted by earlier investigators: (1) blending; the cells are separated mechanically by means of a small electric blender and crudely disintegrated, (2) maceration; the fragmented tissues are macerated by alternate treatments with dilute alkali and dilute acid, (3) mounting on sheet meshes; after washing of the macerated cells, the cell suspensions are dropped on collodion-coated grid meshes and air-dried, (4) shadowing; materials on the grid meshes are shadowed by any one of Cr, Pt or U. This is a series of technique to observe the shadowed materials directly by means of electron microscopy. Thereafter, another method[1] was developed, that is, ultra-thin sections were delignified and embedded in methacrylate resin, then deembedded materials were shadowed by Pd-Au alloys. STERLING and SPIT[2] demonstrated the crossed fibrillar structure in the developing fiber of *Asparagus*; 2~5 $\mu$m sections of parafin embedded materials were deembedded, and their replicas were prepared by shadowing and carbon-backing. Any of these are sort of direct carbon replica technique, and CÔTÉ[3] pointed out that the direct carbon replica technique is a simple, nondistorting, reproducible and highly reliable one in representing fine details.

On the other hand, the preparation technique has been improved to preserve tissues in the more natural condition. Particularly, attempts to use freeze drying methods, solvent exchange drying methods and critical point drying methods have been made to observe the structure of pit membranes in green conditions with variable results by THOMAS[4,5], THOMAS and NICOLAS[6], IMAMURA *et al.*[7], SACHS and KINNEY[8]. These are, however, mainly used as pretreatment for scanning electron microscopy. NORBERG[9] presented a new method for investigating wet wood fiber surfaces; the fibers are ultrarapidly frozen at very low temperature in liquid nitrogen; and frozen specimen is then kept at about −60°C during a freeze drying, metal shadowing and carbon coating process. This is, however, a sort of freeze drying at low temperature.

We must await the following technique to prepare successfully the hydrated

materials without drastic drying.

Freeze etching, which is the more improved technique, has been widely applied to observe surface and three-dimensional structures of the organelles of lower plant[10~15] as well as higher plant[16~18] cells. Since the first observation of Moor and Mühlethaler[19] that the plasmalemma carries hexagonal arrangements of particles which are, according to them, apparently involved in the production of the glucan fibrils of the cell wall of yeast, investigators have interested in the plasmalemma surface relating with the synthesis of wall fibrils[14,20~24]. This technique, however, has not been used as a mean to investigate the organization of lamellae which compose the cell wall of higher plants and algae, except only brief descriptions by CHAFE and WARDROP[25], PENG and JAFFE[26], ROBINSON and PRESTON[27], and BROWN and MONTEZINOS[11].

This chapter discusses the significance of the freeze etching technique as compared with the ordinary replicating ones in investigating the structure of cell walls of cortical parenchyma of poplar.

### 1.2 Nature of "fibrillar structures" on the plasmalemma

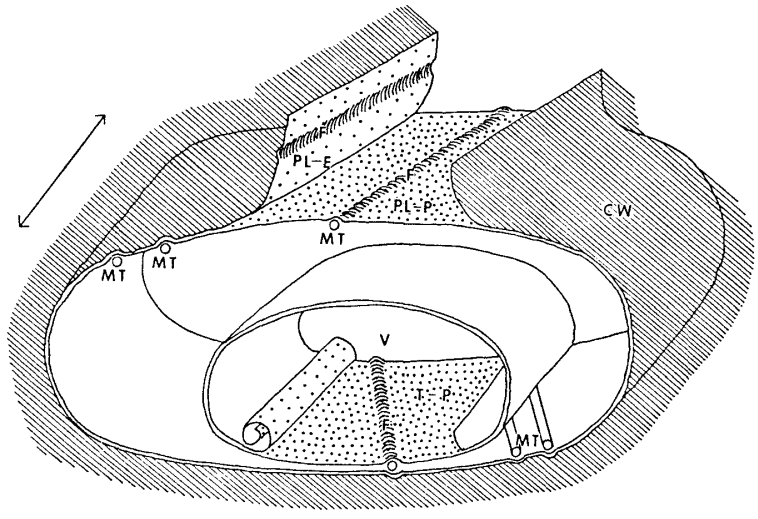
The occurrence of "fibrillar structures" on the plasmalemma surface must be noticed because they seem to reflect the orientation of microtubules as will be discussed in the followings. It is the most interesting and remarkable feature of poplar parenchyma cells observed by the freeze etching technique. On the P-face of the plasmalemma, they are seen as long striations, which are raised slightly above the general background of the plasmalemma (on the right of Fig. 1). On the E-face of the plasmalemma (on the left of Fig. 1), the striations can be seen as long and narrow grooves. The longest "fibrillar structure" reaches more than 13 $\mu$m in length. The "fibrillar structures" are oriented almost in the same direction singly or 2 to 3 in bundles. Furthermore, they run parallel to the wall microfibrils, and a number of randomly distributed particles are found on the "fibrillar structures" as on the general surface of the plasmalemma. When the "fibrillar structures" meet the primary pit fields, the former rarely pass through the latter.

Fig. 2 shows the P-face of plasmalemma and E-face of tonoplast. In this figure, some "fibrillar structures" pass through not only the E-face of the plasmalemma but also the P-face of the tonoplast. They are seen as concave striations on the former and convex ones on the latter. The causative original structure of the "fibrillar structures" may be located in quite a narrow space of cytoplasm sandwiched between the plasmalemma and tonoplast. In fact, when observed by thin sectioning methods, such a narrow space is often observed[28]. Hence, the causative original structure of a "fibrillar structure" is considered to be a microtubule.

The "fibrillar structures" could be presupposed as follows: (1) microtubules

*in situ*, (2) microfibrillar precursors, or (3) the imprint of underlying microtubules on the plasmalemma. The first interpretation is questionable by the fact that the "fibrillar structures" occur on the same plane where the impressions of primary pit fields can be seen, that is, on the plasmalemma surface. The second is not plausible because Fig. 2 substantiates that the causative original structure of the "fibrillar structures" could possibly occur between the plasmalemma and the tonoplast. The results of this investigation support the third one; the "fibrillar structures" are the imprint of underlying microtubules on the plasmalemma surface.

The problem is raised as to how the microtubules can be replicated on the plasmalemma surface and sometimes on the tonoplast in spite of a little separation between the microtubules and the plasmalemma. At first, it was presumed that the plasmalemma was depressed into the cytoplasm during the sublimation of ice or etching. This view, however, is untenable since the "fibrillar structures" can be seen on the plasmalemma even by the freeze fracturing alone without further etching. The "fibrillar structures" could be considered as the protruding of plasmalemma positioned by microtubules toward the cell wall. This situation may be well illustrated schematically as Text-Fig. 1. The planes shown by PL-P and PL-E in this figure correspond to those in Fig. 1, and the planes shwon by PL-E and T-P in this figure correspond to those in Fig. 2.




Text-Fig. 1. Diagrammatic illustration of the occurrence of the "fibrillar structures" both on the plasmalemma and tonoplast.

NORTHCOTE and LEWIS[22] reported in their freeze etching investigation of pea root tips that microtubules appeared at the fractured cytoplasmic plane immediately under the plasmalemma. The figure obtained by them, however, seems to show the imprint of underlying microtubules on the plasmalemma. In their figures, the

plane on which "microtubules" appear can be taken as the plasmalemma surface and the plane on which no "microtubules" appear can be taken as the fractured cytoplasmic plane. This is inferred from the basis on the observation that the former plane has the smoother appearance, which is characteristic of the freeze etched surface of the plasmalemma, and the fractured cytoplasmic plane has the coarser appearance, which is characteristic of the deeply etched groundplasm. Accordingly, the previous observations reported by NORTHCOTE and LEWIS[22] and NORTHCOTE[29] on the possible appearance of microtubules at the fractured cytoplasmic plane inside the plasmalemma are questionable.

If the present view is admitted, some invaginations of the plasmalemma are expected to be seen immediately outside the microtubules also by the observation of ultra-thin sections. No evidence to support such invaginations of plasmalemma, however, has been obtained by sectioning techniques. There might be some coats around the periphery of microtubules which are transparent to electron beam.

### 1.3 Comparison of the freeze etching technique with the conventional replicating one

The advantage of the conventional replicating techniques including freeze drying followed by shadow-casting one is that the investigation of microfibrillar orientation of a primary wall is useful in a point where the oriented microfibrils are observable in a large area. The following disadvantages, however, are noticed in these techniques[30].

(1) Wavy pattern of microfibrils is sometimes observed as artifacts. Such phenomena are also seen in the reports of ROELOFSEN and HOUWINK[31], WARDROP[32], SETTERFIELD and BAYLEY[33], MOOR[34] and IMAMURA *et al.*[7], *etc.*

(2) Three dimensional relationship between each lamella, and among cytoplasm, plasmalemma and cell wall, is not clear.

(3) Randomly oriented microfibrils are observed very often, which may occur because of the displacement of microfibrils during each step of the preparation of materials, especially of "blending" and "maceration"[35].

(4) Bundled microfibrils are frequently observed as artifacts. Similar phenomena can be seen in the reports of HOUWINK and ROELOFSEN[36], BOHMER[37], WARDROP[32,38] and IMAMURA *et al.*[7], *etc.*

(5) Tissue shrinkage may occur in any conventional replicating methods during the drying processes.

In contrast to the conventional replicating techniques, the freeze etching technique proved to have many advantages as follows[30].

(1) Highly oriented microfibrils are generally seen in fractured walls of *Pinus, Phaseolus* and *Populus* respectively, while bundled or wavy microfibrils are not observed at all.

These highly oriented microfibrils occurring in primary walls are a characteristic feature which has never been reported. Fixation and glycerol impregnation are processes which may induce structural modifications as artifacts, if any, by this technique. It is incredible that either of these processes may introduce any artificial changes in the arrangement of wall microfibrils.

(2) Three dimensional relationships between respective lamellae, and among cytoplasm, plasmalemma and cell wall are clearly recognized.

(3) Orientation of microfibrils inherent to each lamella is clearly discernible even if lamellae is piled up in a complex manner.

(4) Polylamellate structure of a parenchyma cell wall is clearly seen. In another case, epidermal cell walls of *Phaseolus* observed by the freeze etching technique also show a polylamellate structure in which it is clearly seen that lamellae tend to be thinner near the cuticle.

(5) It is possible to determine the width of microfibrils *in situ*. Each microfibril which does not suffer from any drastic chemical treatment or severe drying is revealed by the freeze fracturing.

However, it is not so easy to obtain freeze etched replicas of plant tissue because of the greatest difficulties encountered in the final step of releasing the replica from the underlying tissue. The carbon-platinum replicas are extremely fragile and brittle and, therefore, cannot be directly manipulated without the possibility of their breakage.

Hence, the freeze etching technique is quite time consuming in the application for investigating the cell wall organization of plant tissues. In spite of the shortcoming mentioned above, the freeze etching technique is superior to the conventional replicating technique in reducing the artifacts and obtaining highly reliable representation of the microfibrillar orientation in the more natural condition.

### 1.4 Observation by the freeze etching technique without any pretreatment

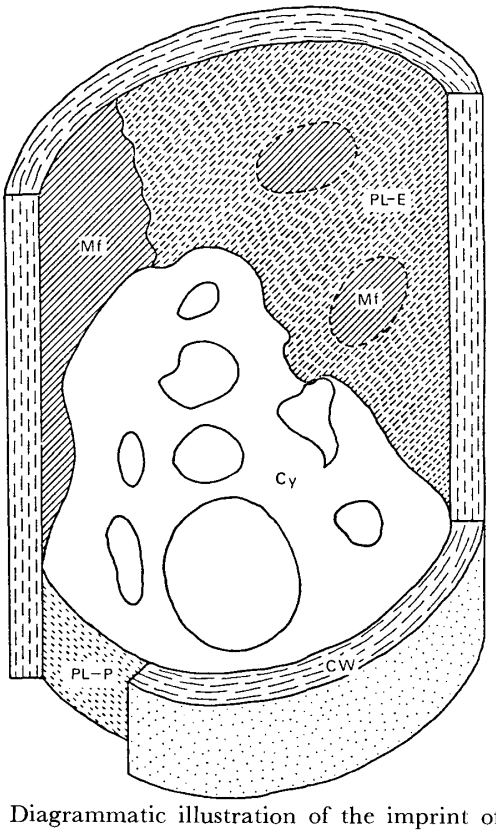
In order to clarify the details of the synthesis and incorporation of cell wall precursor, one must obtain informations from the observation of quite a narrow region sandwiched between the plasmalemma and the cell wall. For this purpose, the application of the freeze etching technique without pretreatment is thought to be most profitable. In fact, many investigations have been presented to clarify the orientation and synthesis of microfibrils by the application of this technique[13,26,39~41].

The cytoplasm of the shoot frozen without any pretreatment are found to be distorted by intracellular ice crystals. However, the plane of fractured plasmalemma are rarely affected by ice formation. Many primary pit fields which contain a large number of plasmodesmata are seen on the E-face of fractured plasmalemma. Mem-

brane-associated particles are also observed on both the P- and E-faces of fractured plasmalemma. However, there are not any difference in quantities and arrangement of the particles on both planes, which is different from the images commonly obtained by the pretreatment with glutaraldehyde and glycerol solution.

On the E-face of plasmalemma, many convex striations running parallel to one another can be seen (Fig. 3), while on the P-face of plasmalemma many concave striations running parallel to one another can be seen (Fig. 4). These are the impressions of microfibrils just on the plasmalemma. In glutaraldehyde-fixed and glycerol-pretreated materials, on the other hand, any such striations can never be seen except the "fibrillar structures" which are the impressions of the underlying microtubules on the plasmalemma (Figs. 1 and 2). Consequently, as suggested by PENG and JAFFE[26] and WILLISON[40,41], the impressions of microfibrils on the plasmalemma may occur under normal growing conditions by turgor pressure pressing the plasmalemma of a cortical parenchyma cell of poplar tightly against the surrounding cell wall. If it is so, these facts may indicate that, once the plasmalemma is pretreated with the fixing fluid and/or glycerol, it is deformed and is detached more or less from the cell wall.

Plasmalemma remained to be pressed so tightly to the underlying cell wall that




Text-Fig. 2. Diagrammatic illustration of the imprint of microfibrils on the plasmalemma.

the disposition of microfibrils is seen through some regions (short arrows) in which plasmalemma is happened to be torn off (Fig. 5). Such situation is well illustrated schematically in Text-Fig. 2. In this way, freeze-fracturing without any pretreatment showed the regularly oriented and compact microfibrils immediately under the plasmalemma. This is a strong evidence supporting that there is no intermediate region with loosened and random microfibrils close to the plasmalemma. Besides, the freeze etching technique without any pretreatment shows more intact orientation of microfibrils in a lamella just outside the plasmalemma.

## 2. The structure of cortical parenchyma cell walls during elongation growth

### 2.1 Early informations on the wall structure of parenchyma cells

The optical properties of the growing cell wall, that is, the orientation of microfibrils has been studied increasingly since the applications of polarization microscope. Under crossed nicols, the primary wall always appears to be positively birefringent when viewed in the plane of wall while in growing cells it is, with very few exceptions, negatively birefringent with reference to the axis of the cell[35]. According to BONNER[42], transverse microfibrils are continuously laid down on the inner surface of the primary wall to compensate the change into longitudinal direction of the older microfibrils. On the other hand, FREY-WYSSLING[43] explains that no change in the direction of microfibrils is taken place with a loosening of the joints after which the microfibrils may slide further apart during longitudinal growth. Thus, in both cases, there is a constant negative birefringence during growth. Although the gross orientation of microfibrils can be deduced by means of the polarization microscope, it must await the advance of electron microscopy to investigate the individual microfibril orientation in a lamella.

Since the earliest attempt[44] to study the structure of the primary wall by means of the electron microscope, a great many investigations have been presented on the structure of primary walls of parenchyma cells[31,32,36,45~49]. Previous investigators have shown that thin primary walls of parenchyma cells have an inner region of predominantly transversely oriented microfibrils bounded outside by a region with microfibrils having an irregular transition to a longitudinal orientation[35,50~53]. These results have been well explained by the multinet growth hypothesis as originally presented by ROELOFSEN and HOUWINK[31]. However, it seems that there are such difficulties to explain the wall extension by the hypothesis that microfibrillar orientation in epidermal wall is reported to be partly parallel to the longitudinal cell axis, (2) almost all of the parenchyma cells have rib thickenings with longitudinal microfibrils and sometimes (3) primary walls consist of crossed lamellate structures. When CHAFE and WARDROP[54~56] observed collenchyma and epidermal cell walls in

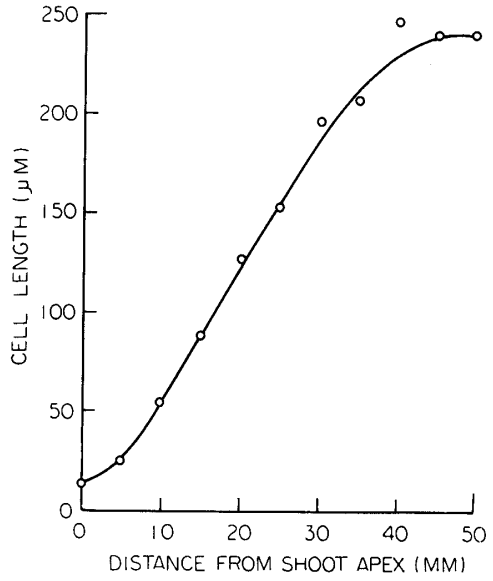
the petioles of some species, they encountered the difficulty in explaining the wall structure by the multinet growth hypothesis and presented the modificatioonf the hypothesis. Furthermore, they inferred that the wall structure of parenchyma cells is similar to that of collenchyma and epidermal cell walls[55]. Recently, by the application of ultracryotomy with negative staining and cytochemistry, ROLAND *et al.*[57,58] investigated the architecture of parenchyma cell walls of some species and observed well ordered microfibrils and no progressive change of them from the transverse orientation near the plasmalemma. Based on the results obtained, they postulated "ordered fibril hypothesis".

In spite of many investigations mentioned above, there is a great discrepancy of informations obtained on the structure of parenchyma cell walls. This chapter discusses the wall structure of cortical parenchyma cells of poplar during the elongation growth.

### 2.2 The change of wall thickness

The average length and breadth of the cortical parenchyma cells during shoot elongation are recorded in Table 1, while detailed values are plotted in Text-Fig. 3. As is known from the table and the text-figure, cell elongation of a poplar shoot continues until newly formed cells are moved approximately 45 mm far from the shoot apex. On the other hand, the radial breadth of cortical parenchyma cells ceases to expand at the position of about 10 mm or so from the shoot apex.

As for the change of the wall thickness of cortical parenchyma cells (corresponding to third or fourth cells from epidermis), estimation is made on the positive prints




Text-Fig. 3. Cell length of cortical parenchyma of poplar measured at various stages.

Table 1. Dimensions of cortical paernchyma cells of a poplar shoot at various distances from the apex.

| Stage of growth of cells Distance from shoot apex (mm) | Average cell length ($\mu$m)* | Average cell breadth ($\mu$m)** |
|---|---|---|
| 0 | 14 | 27 |
| 5 | 26 | 39 |
| 10 | 55 | 47 |
| 15 | 89 | — |
| 20 | 128 | 47 |
| 25 | 154 | — |
| 30 | 197 | 53 |
| 35 | 207 | — |
| 40 | 247 | 49 |
| 45 | 240 | — |
| 50 | 240 | 51 |

* Average of 50 measurements estimated under photo microscope.
** Average of 10 measurements estimated under photo microscope.

Table 2. Wall thickness of cortical parenchyma cells and epidermal cells of poplar during cell elongation.

| Specimen number | Cell type | | Wall thickness at various distances from the shoot apex ($\times 0.16\ \mu$m) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| No. 1 | Parenchyma cell* | | 0.5 | 1.5 | 1.5 | 1.9 | 2.4 | 3.4 | 3.8 | 3.7 |
| | Epidermal cell | outer wall** | 2.4 | 3.9 | 4.5 | 5.0 | 8.6 | 12.8 | 13.6 | 13.4 |
| | | inner wall* | 0.8 | 1.3 | 1.5 | 1.4 | 2.0 | 2.8 | 3.5 | 3.4 |
| | | side wall* | 0.3 | 0.6 | 0.7 | 0.8 | 1.3 | 2.2 | 3.0 | 2.9 |
| No. 2 | Parenchyma cell* | | 1.2 | 1.4 | 1.5 | 2.1 | 2.2 | 3.0 | 3.6 | 3.9 |
| | Epidermal cell | outer wall** | 2.5 | 3.6 | 4.0 | 4.7 | 6.2 | 8.9 | 13.3 | 13.0 |
| | | inner wall* | 1.2 | 1.2 | 1.3 | 1.7 | 1.5 | 3.1 | 3.6 | 3.8 |
| | | side wall* | 0.6 | 0.6 | 0.7 | 0.8 | 0.9 | 2.0 | 3.2 | 2.8 |
| No. 3 | Parenchyma cell* | | 1.2 | 1.6 | 1.8 | 2.0 | 2.8 | 3.3 | 3.7 | 3.5 |
| | Epidermal cell | outer wall** | 3.8 | 4.0 | 4.7 | 4.3 | 7.8 | 7.9 | 13.4 | 13.5 |
| | | inner wall* | 1.4 | 1.5 | 1.6 | 1.5 | 2.2 | 2.2 | 3.5 | 2.9 |
| | | side wall* | 0.6 | 0.7 | 0.8 | 0.8 | 1.8 | 1.6 | 2.5 | 2.6 |
| Distance from the shoot apex (mm) | | | 0 | 5 | 10 | 20 | 30 | 50 | 70 | 100 |

* Average of 10 measurements estimated from electron micrographs.
** Average of 5 measurements estimated from electron micrographs.

about 3 times magnified from the original negative films obtained by the electron microscope. For comparison, the wall thickness of epidermal cells is also estimated. The results obtained are shown in Table 2. In epidermal cells the thickness of outer, inner and side walls is measured respectively. In parenchyma cells the wall thickness is measured on the narrowest point situated between any two of cell corners.

In considering the data of cell elongation (Table 1) and wall thickness (Table 2), it is indicated that the wall thickness of both parenchyma cells (corresponding to third or fourth cell from epidermis) and epidermal cells increases gradually with cell elongation.

### 2.3 Orientation of microfibrils and microtubules

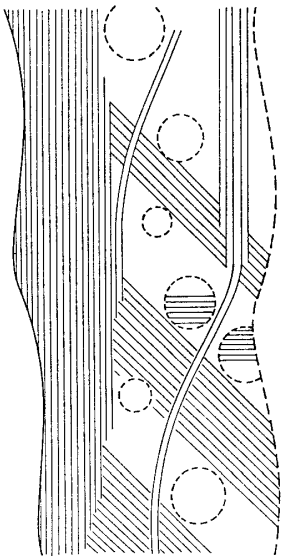
*Observation by the freeze drying and freeze etching techniques*

When investigated by the freeze drying technique, the innermost lamella with random microfibrils is sometimes seen in the walls of elongating parenchyma cells of poplar after plasmolysis in high concentration of sucrose, although it shows variable highly oriented microfibrils when investigated by the freeze etching technique. Recently, however, the present author suggests that highly oriented microfibrils are the common feature of freeze etched walls of primary nature[30]. It seems reasonable to consider that the so called "random microfibrils" may be the result of the displacement of microfibrils due to the repeat of swelling and shrinkage during the preparation of materials by the successive treatments such as freeze sectioning, delignification with acidic sodium chlorite, washing in distilled water, and freeze drying. It should also be noticed that Frei and Preston[59] explained random microfibrils as the results of the loss of turgor due to plasmolysis, when they observed random microfibrils on the innermost surface of the wall of *Chaetomorpha melagonium*. Recently, on the other hand, Roland *et al.*[57] noted that the outermost lamella of the wall of root cap of pea (*Pisum sativum*) showed randomly oriented microfibrils.

The lamellae with above mentioned highly oriented microfibrils are classified into three types.

The first type of these lamellae is that composed of almost transversely oriented microfibrils. Fig. 6, an image obtained by the freeze drying technique, shows such microfibrils which are crossed by the underlying ones nearly at right angles. Transversely oriented microfibrils are also observed in the exposed surface of freeze-fractured wall (Fig. 7).

The second is the lamella composed of almost longitudinally oriented microfibrils. Fig. 8, an image obtained by the freeze drying technique, shows the microfibrils in the innermost surface of the wall running parallel to the main cell axis. These microfibrils are sparsely distributed, enabling the observation of microfibrils within the lamella beneath the surface one, which have an orientation almost transverse to the main cell axis. Longitudinally oriented microfibrils were observed also in the exposed surface of fractured wall (Fig. 9). Fig. 11 shows a remarkable evidence which is difficult to explain by the hypothesis. This side of the figure shows the inner face of the cell wall. Text-Fig. 4 is a schematic representation of Fig. 11. There are three types of microfibrillar orientation. Transverse microfibrils are the oldest,



Text-Fig. 4. Diagrammatic illustration of Fig. 11, showing transverse, oblique and longitudinal microfibrils, respectively.

while the longitudinal ones are the youngest in the sequence of microfibrillar deposition. According to multinet growth hypothesis, the older the microfibrils the more longitudinal their orientation become; microfibrils direct more longitudinal in outside than inside within a cell wall. This situation is not compatible with that of the result in Fig. 11. Because multinet growth hypothesis implies that the transverse orientation of microfibrils on the inner surface of the cell wall must gradually be shifted via an isotropic into a more nearly axial orientation toward the outer surface, it is difficult to explain either the crossed structures of microfibrils or the occurrence of the longitudinally oriented microfibrils not only in the innermost lamella of but also in the fractured plane within the parenchyma cell wall by this hypothesis. Longitudinal microfibrils, however, did not occur so frequently as the first and third types in freeze drying and freeze etching preparations.

The third type is the lamella in which the microfibrillar orientation is oblique to the main cell axis (Fig. 10, an image obtained by the freeze drying technique). Microfibrils of the innermost lamella are crossed with reversely oblique ones of the underlying lamella. Recently, VEEN[60] observed oblique microfibrils in parenchyma cells of pea stem during longitudinal growth by polarization and electron microscope. The oblique microfibrils in the walls of young cells of etiolated pea shoot apex have been also observed by RIDGE[61]. They interpreted that a thin layer of oblique microfibrils was situated at the outer surface of the cell wall, and suggested that this layer arose from rotation of the originally transverse microfibrils as a result of cell extension. Oblique microfibrils are also observed in the exposed surface of the fractured walls

(Figs. 12 and 13). Oblique microfibrils in Fig. 12 show both compact and not so compact or sporadic ones. This situation seems to be in quite a good correspondence with the view of trellis-like configulation presented by BOYD and FOSTER[62]. In the figure 13, microfibrils of the lamella just under the surface one showed reverse orientation to those of the surface lamella as is seen at the openings of the pit fields. This case could not be explained by rotation of the originally transverse microfibrils due to mechanical stretching of the wall as suggested by VEEN[60] and RIDGE[61].

In any case, oblique microfibrils are commonly found not only in the innermost wall surface but also throughout the extending walls of cortical parenchyma cells of poplar. This was further strengthened by the occurrence of oblique microtubules, which will be mentioned in the followings. On the basis of the above observations, cell walls of poplar parenchyma during elongation growth may be composed of the lamellae of all the above mentioned three types of microfibrillar orientation, although the frequency of the occurrence of each type is somewhat different. This view is in good agreement with the results that three types of microfibrillar orientation have been observed in radially enlarged parenchyma cells of coumarin- and colchicine-treated pine seedlings[63].

*Observation by the freeze etching without pretreatment*

In the preceding chapter, the author suggests that the freeze etching technique without pretreatment shows more intact and highly reliable orientation of microfibrils in the innermost lamella by the observation of their impressions on the plasmalemma possibly due to turgor pressure(Text-Fig. 2). Therefore, the orientation of the most recently deposited microfibrils can be seen with high reliability by the freeze etching technique without pretreatment.

Most of the newly deposited microfibrils were oriented perpendicular to longitudinal cell axis with highly ordered pattern (Fig. 14). This type of orientation could frequently observed in the inner cortical parenchyma cells. However, the oblique orientation of newly deposited microfibrils was also observed (Fig. 15). Sometimes, the newly deposited microfibrils were oriented parallel to the longitudinal axis (Fig. 16). In this figure, the primary pit fields are circled by dotted line and show an ellipse; the major axis of the ellipse corresponds to the direction perpendicular to the cell axis. The latter two types of orientation, namely oblique and longitudinal, could frequently be seen in the outer cortical parenchyma cells. In Fig. 17, though orientation of microfibrils of the surface lamella was oblique, microfibrils of the lamella immediately under the surface lamella were perpendicular to longitudinal cell axis. This case shows that the angle of newly deposited microfibrils to longitudinal cell axis is smaller than that of underlying microfibrils to longitudinal axis, which is inconsistent with the multinet growth hypothesis implying that the transverse orientation

of microfibrils on the innermost lamella of the cell wall must gradually be changed through random into a more nearly axial orientation toward the outer surface.

Orientation of microfibrils in secondary wall thickening is thought to be highly correlated with the orientation of microtubules[21,64~77]. Although the above view is roughly accepted also in the primary wall thickening, there are some variations suggesting the incompatibility[78~80]. In order to examine the correlation between microtubules and microfibrils in the elongating parenchyma cells, longitudinally sliced sections have been observed. The results showed transverse, longitudinal and oblique microtubules respectively, coinciding with the same orientations of microfibrils in the wall[81]. The more strict orientation of microtubules could be presented by the orientation of "fibrillar structures" observed by the freeze etching technique on the plasmalemma surface[18,28], because these "fibrillar structures" were interpreted as the imprint of underlying microtubules on the plasmalemma surface[28]. The present investigation showed three types of the imprint of microtubules by the same technique; one was perpendicular (Fig. 18), another parallel (Fig. 19) and the other oblique (Fig. 20) to the main cell axis. Therefore, it is considered that three types of microtubule orientation occur in elongating cells of cortical parenchyma of poplar, although longitudinally oriented microtubules were scarcely observed.

The past observations on cells during primary wall growth indicate that the microtubules next to the lateral walls are always oriented transversely like the newly deposited microfibrils[73,75], except the observations of axially oriented microtubules in the cytoplasm next to the lateral wall in the root hair of radish[78] and in collenchyma cells of *Apium*[25]. The present results, however, showed the occurrence of not only the transverse microtubules, but also the longitudinal and oblique ones in elongating parenchyma cells of poplar. Besides, the three directions of microfibrillar orientation corresponded to the three directions of microtubule orientation respectively.

### 2.4 The occurrence of crossed polylamellate structure

It is well known that crossed lamellate structure occurs in the secondary wall in a variety of cell types of higher plants[82~86]. Some authors noted previously the same structure also in the cell wall of primary nature[2,38]. More recently, the crossed lamellate structure in the primary wall has been increasingly noticed in a variety of cell types such as collenchyma[54,56,57], epithelial cell[87], epidermal cells[55,88], sieve cells[89,90], ray parenchyma cells[91] and cortical parenchyma cells[57,58,63,92]. These evidences are based mainly on electron micrographs of thin sections shadow-casted with metals after removal of the embedding medium. This is one of the most useful technique in investigating microfibrillar orientation throughout a cell wall. Fig. 21 shows the area in which three single walls ($W_1$, $W_2$, $W_3$) are conjugated. Crossed microfibrillar structure is shown in $W_2$ which is cut almost parallel to the tangential

direction of a cell. Fig. 22 shows a double cell wall cut almost parallel to the main cell axis. The lamellate structure is not so distinct in Wa because the wall is cut perpendicular to the tangential direction. On the other hand, Wb which is sliced obliquely to the tangential direction, shows typical crossed polylamellate structure in which lamellae having a generally longitudinal orientation of microfibrils (L) alternate with lamellae having a generally transverse orientation (T). In this case, "longitudinal" and "transverse" do not necessarily mean their strict directions.

As is already shown in Table 2 and Text-Fig. 3, cortical parenchyma cells are under elongation growth at least until they receded 45 mm from the shoot apex. In order to clarify whether some changes occur in cell wall organization of the cortical parenchyma cells during their elongation, longitudinal sections obtained from one shoot at the distance of 0 mm, 5 mm, 10 mm (Fig. 23), 20 mm (Fig. 24), 30 mm and 50 mm from the shoot apex are shadow-casted with metals after removal of the embedding medium and investigated at ultrastructural level. These figures show crossed polylamellate structures in almost all of the stages. Even in an earlier stage of cell elongation, the cell wall has a typical crossed microfibrillar structure. However, the outermost wall seems to have longitudinal microfibrils. This situation is well explained from the observation of the oblique section of a single wall (Fig. 25). The wall possesses crossed polylamellate structure in almost all of the wall area from lumen to outer wall except the innermost wall layer in which microfibrils oriented in a longitudinal direction.

In the preceding experiments, it is indicated that the freeze etching technique is useful in demonstrating the polylamellate structure of cell walls. In the more recent investigation, the cell wall of cortical parenchyma of *Populus, Phaseolus* and *Morus* is shown to have polylamellate structures on the observation of cross fractured walls[92]. Parenchyma cell walls of *Populus* and *Morus* consist of about 8 lamellae, while the wall of *Phaseolus* parenchyma cells consists of more than 15 lamellae. Fig. 26 shows an exposed surface of a longitudinally fractured wall of a *Populus* cell. The lamellae are recognized as such that the orientation of microfibrils is more or less parallel to the main cell axis (short arrows), although the lamellae in which the microfibrillar orientation is almost transverse (long arrows) are predominant throughout the wall thickness. The microfibrillar orientation of these lamellae cross at about 90°. Fig. 27 shows a longitudinally fractured wall of a parenchyma cell in the cortex of *Phaseolus*. Some impressions of primary pit fields are seen on the plasmalemma plane; this is thought to be characteristic of the primary wall. A higher magnification of the inlet of Fig. 27 is shown in Fig. 28. The lamellae, in which the microfibrillar orientation is perpendicular to the main cell axis (short arrows), alternate with those having a microfibrillar angle of about 45° to the main cell axis

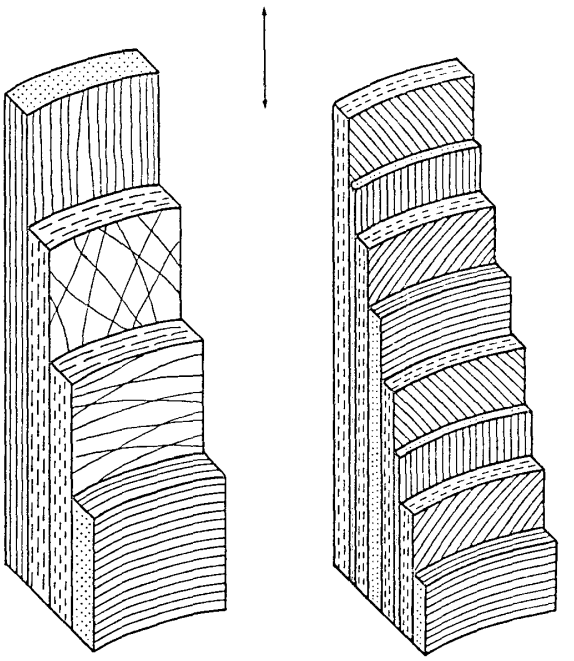
(long arrows). Thus, the microfibrillar orientation of the two adjacent lamellae cross at 45°.

In the case of *Morus* parenchyma cells, a variety of interlamellar crossing angles of microfibrillar orientation is observed. Fig. 29 shows the exposed surface of the fractured wall of a *Morus* parenchyma cell. In this figure, the lamellae A is deposited on B, which is deposited on C. The microfibrillar orientation of both A and C (short arrows) is the same, and the lamella B (long arrows) is shown to be crossed with either lamella A and C in a smaller angle than 90°.

The occurrence of crossed polylamellate structure during elongation of cortical parenchyma cells is further confirmed by the observation of longitudinally fractured plane of the walls; Fig. 30 shows crossed lamellate structure in which microfibrils oriented mainly in two directions as indicated by short arrows.

The presence of the crossed polylamellate structure indicates that the structure of the primary wall is not merely a passive result due to physical forces. Multinet model for wall growth as originally presented by ROELOFSEN and HOUWINK[31] is too simple to account for the following observation in the parenchyma cells discussed here. That is, (1) microfibrils of primary walls are highly oriented ones, (2) the cell wall during cell elongation has crossed polylamellate structure which is seen in the wall of any stage of cell elongation and (3) oblique orientation of microfibrils as well as transverse and longitudinal one is observed throughout the cell wall, especially in the innermost lamella of the walls of elongating cortical parenchyma cells of poplar. Whether the orientation of microfibrils become transverse, oblique or longitudinal may be determined at their deposition in the course of cell development and may not be reoriented thereafter. Accordingly, such a view that the orientation of microfibrils may not be influenced drastically by the extension of the wall should be taken into consideration instead of multinet growth theory in the case of present materials.

Although the present results are roughly illustrated on the basis of "ordered fibril hypothesis" of ROLAND *et al.*[57], some modifications are needed for the hypothesis because of too much simplicity of it. First of all, the presence of oblique microfibrils in addition to transverse and longitudinal ones should be taken into consideration. Text-Fig. 5-b is the tentative illustration of modified "ordered fibril hypothesis", compared with multinet growth hypothesis of Text-Fig. 5-a. Each lamella in the Text-Fig. 5-b may be alternative with a multi-lamellae, depending on the cell type and the position in the tissue. Besides, the lamellae with longitudinal microfibrils may occasionally be seen. On the basis of the modified "ordered fibril hypothesis", cell wall growth may be explained as follows: (1) the most probable structural change may be performed by the sliding between lamellae during cell elongation; (2) the intralamellar structural changes may also be presumed within transverse, oblique



Text-Fig. 5. Comparison between the multinet growth hypothesis (A, left) and the modified "ordered fibril hypothesis" (B, right). The direction of cell elongation is indicated by a double-headed arrow.

and longitudinal lamellae; (3) in transverse and oblique lamellae, adjacent microfibrils are separated singly or in groups during the extension of the lamellae, resulting in gaps similar to "trellis-like configurations" as has been presented by BOYD and FOSTER[62], and microfibrils in a lamella are readjusted by the sliding of themselves so as to fill up the gaps or spacing resulting from the wall extension and (4), in longitudinal lamella, microfibrils fill up the spacing resulting from the wall extension by their longitudinal sliding.

## 3. Microfibrillar orientation of swollen cells of coumarin- and colchicine-treated pine seedlings

### 3.1 Early informations on the cell swelling

According to the multinet growth hypothesis, cells enlarge predominantly in a longitudinal direction because of the restraining influence of newly deposited and radially oriented microfibrils. However, if a cell is treated by some chemicals such as ethylene, supraoptimal IAA, coumarin and colchicine, radial expansion of the cell is induced. Therefore, if a cell is to expand radially under the influence of such chemicals, newly deposited microfibrils should not be oriented in a radial direction based on the multinet growth hypothesis.

WARDROP[47] observed that the initial transverse orientation of microfibrils of parenchyma cells radially enlarged by the treatment with colchicine tended to be

maintained in the outer surface, and suggested that the final microfibril orientation on the outer surface of the wall is determined by the extent and polarity of its surface growth. On the other hand, PROBINE[93] observed bands of longitudinal microfibrils on the outside of the cell wall of the sub-apical sections of pea epicotyls treated with a solution containing indole acetic acid (IAA) and benzimidazole. APELBAUM and BURG[94], SARGENT *et al.*[95,96] investigated the orientation of microfibrils in the sub-apical region of pea seedlings treated with ethylene, and observed that the microfibrils at the inner surface of the wall were oriented predominantly in a longitudinal direction. They suggested that ethylene induced the deposition of longitudinally oriented microfibrils which restrict the cell to expand longitudinally, but allow to swell radially. Recently, SHIBAOKA[97] and HOGETSU *et al.*[98] observed that the orientation of wall microtubules changed to the longitudinal direction during kinetin-induced cell swelling, and suggested that longitudinally oriented microfibrils must be deposited.

The relationship between chemically induced radial expansion of parenchyma cells and the orientation of cellulose microfibrils has not been well demonstrated. In order to clarify the above problem, this chapter discusses the orientation of microfibrils on the inner surface of the walls of coumarin- and colchicine-treated cells of pine seedlings. The occurrence and distribution of microtubules in those cells are also discussed.

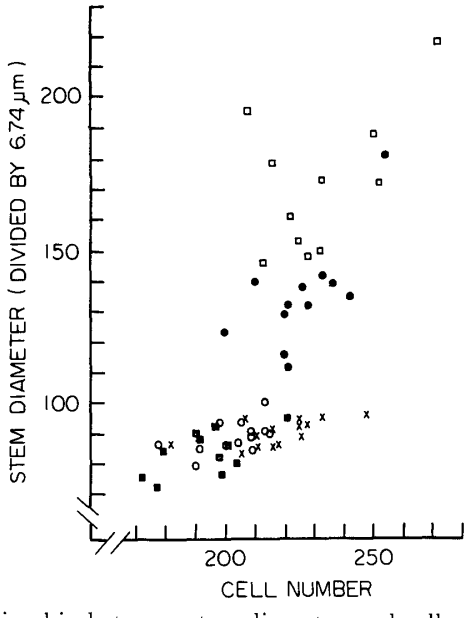
### 3.2 Cell swelling of pine seedlings

Before entering into the above-mentioned problem, we must clarify the nature of radial enlargement of pine seedlings treated with coumarin and colchicine.

Pine seedlings (*Pinus thunbergii* Parl.) were grown on wet vermiculite in a dark room at 28°C. When the hypocotyl reached 1～2 cm in length, the seedlings were transferred to small glass tubes containing $7\times10^{-3}$ M coumarin or $6\times10^{-3}$ M colchicine and grown further in a dark room. The concentration of coumarin and colchicine were selected on the basis of the results obtained from preceding investigation[99], which showed that seedlings treated with these concentrations expanded radially without much elongation.

For determination of increase in stem diameter, transverse sections of the seedlings which showed some tissue swelling due to $7\times10^{-3}$ M coumarin, and sections of seedlings with tissue swelling due to $6\times10^{-3}$ M colchicine solution, as well as the sections of non-treated (control) seedlings, were cut and subjected to the microscopical estimation. The widest and the narrowest diameters for each of the above sections of control and treated seedlings were measured using a micrometer attached to a light microscope. The average of these two values was taken as representative of the stem diameter. Whether or not the growth in diameter involves the increase of cell numbers was investigated by counting epidermal cells in the cross sections.

The diameters of seedlings treated with either coumarin or colchicine greatly exceeded those of the control, although the diameters of coumarin-treated seedlings were much smaller than those of colchicine-treated ones[99]. The increase of diameter in chemically treated seedlings involved the increase in diameter of each structural element such as epidermis, cortical parenchyma, sieve elements, vascular parenchyma and pith parenchyma, among which cortical parenchyma especially was found to contribute most to the increase in diameter. To determine whether the radial enlargement of the stem is due to the increase in cell numbers produced by cell division or the increase in cell volume, the stem diameters and the numbers of epidermal cells in the transverse sections of the controls, coumarin-treated and colchicine-treated seedlings were estimated (Text-Fig. 6). Although the number of epidermal cells was nearly the same in all three cases, the diameter of the colchicine-treated seedlings was a little larger than that of the coumarin-treated seedlings, the diameter of which was much greater than that of the controls. It is concluded, therefore, the radial enlargement of pine seedlings is caused not by the increase of cell number but by the increase of cell volume.

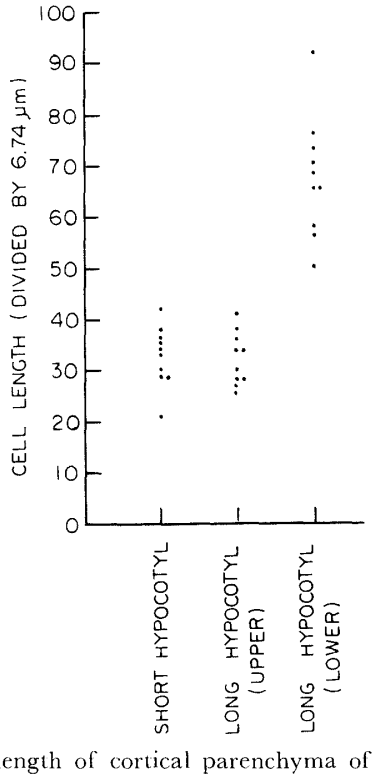



Text-Fig. 6. Relationship between stem diameter and cell number of epidermal cells of control, coumarin ($7 \times 10^{-3}$ M)- and colchicine ($6 \times 10^{-3}$ M)-treated seedlings. Twelve seedlings were used for estimation in each case. The figure on the ordinate shows mean stem diameter divided by 6.74 ($\mu$m). □: colchicine-treated seedlings, ●: coumarin-treated seedlings, ■, ×, ○: control seedlings, showing a short hypocotyl (■), the upper portion of a long hypocotyl (×) and the lower portion of a long hypocotyl (○).

### 3.3 Microfibrillar orientation of swollen cells

*Control cells.*

Text-Fig. 7 shows comparative data on cell length of cortical parenchyma in the sub-apical regions of non-treated pine seedlings before and after elongation growth. Cell length in the short hypocotyl region is similar to that in the lower region of long hypocotyls. The sub-apical regions of two-day old seedlings, that is, short hypocotyls are considered to be under the process of elongation growth. Thus, the orientation of microfibrils of the parenchyma cells in elongating zones is regarded to reflect that of microfibrils of primary walls as defined by WARDROP[48].




Text-Fig. 7. Cell length of cortical parenchyma of a short hypocotyl, upper portion of a long hypocotyl, and lower portion of a long hypocotyl. Each spot shows the mean length of 50 cells. The figure on the ordinate shows mean cell length divided by 6.74 ($\mu$m).

Although transverse, longitudinal and oblique microtubules are seen on the inner surface of the walls of cortical parenchyma cells of poplar, it is not clear whether control or non-treated cells of pine have a similar orientation of microtubules.

The preceding investigation[63] showed the occurrence of longitudinally oriented microtubules as well as transversely oriented ones, although the microtubules were distributed sparsely. Therefore, it is suggested that the deposition of microfibrils parallel to the longitudinal cell axis may actually occur at the inner surface of the

walls of non-treated parenchyma cells.

In order to confirm this point, the orientation of microfibrils at the innermost lamella of parenchyma cell walls was investigated[63]. As has been generally recognized, transversely oriented microfibrils are predominantly observed in the walls of control parenchyma cells. However, as is shown in Fig. 31, almost longitudinally oriented, microfibrils are also observed. Therefore, the orientation of microtubules in control cells of pine seedlings is the same as that of cortical parenchyma cells of poplar.

*Coumarin-treated cells.*

It is quite interesting to see whether or not the cell wall organization is changed during the coumarin-induced swelling of parenchyma cells. The replicas of the innermost surface of the walls of coumarin-treated cells show a variety of microfibrillar orientation, such as a random network of microfibrils and highly oriented, crossed microfibrillar structures. In Fig. 32, microfibrillar orientation of the innermost lamella is parallel to the main cell axis, and microfibrils of the lamella immediately under the surface lamella run perpendicular to the main cell axis. In Fig. 34, the orientation of microfibrils is perpendicular to the main cell axis and microfibrils of the lamella immediately under the surface lamella run oblique to the main cell axis. In the case of Fig. 36, microfibrils of the surface lamella run obliquely (at about 45° to the main cell axis). In this figure, microfibrillar orientation of the lamella immediately under the surface lamella is reversely oblique to the main cell axis. As described in the Chapter 1, all the images of cell wall structure obtained by the freeze etching technique show highly oriented microfibrils, and random orientation of microfibrils is not observed at all. Therefore, it should be possible to consider that the random network of microfibrils which is observed by ordinary preparation method in the replica technique may be artificially produced because of the displacement of microfibrils during the preparation of materials. Thus, it is conceivable that the actual orientation of microfibrils in the walls of coumarin-treated cells may be confined to the following three regular types: one runs parallel, another runs oblique and the third runs perpendicular to the main cell axis. Cell swelling agents are generally thought to have the property of changing microfibrillar orientation[60,61,93,94,96,98,100,101], that is, cells expand predominantly in a radial direction because of the restraining influence of newly deposited and longitudinally oriented microfibrils. The evidence from Figs. 34 and 36, however, does not support the above view, since the inner surface of parenchyma cell walls show a crossed microfibrillar structure. In this case, the microfibrillar orientation of the innermost surface is almost perpendicular to the main cell axis.

Figs. 33 and 35 show a cross sectional view of coumarin-treated cells. As indicated by arrows, longitudinally oriented microtubules (Fig. 33) were frequently

observed together with transversely oriented ones (Fig. 35). The evidence suggests that coumarin-treated cells may induce the deposition of at least two types of differently oriented microfibrils in the inner surface of the wall. Oblique orientation of microtubules, however, was not observed because of the difficulty in distinguishing from the longitudinal one on the image of ultra-thin sections, especially cross sections. Because obliquely oriented microfibrils are observed in the replica of coumarin-treated seedlings, obliquely oriented microtubules should necessarily occur.

*Colchicine-treated cells.*

It is well known that xylem elements differentiated in the presence of colchicine possess abnormal secondary wall thickenings[77,99,102~104]. Parenchyma cells of pine seedlings elongating in the presence of colchicine, however, are not characterized by abnormal wall thickenings, but by radial enlargement. Radially enlarged parenchyma cells of pine seedlings treated with colchicine did not show the occurrence of any wall microtubules immediately under the plasmalemma. Green[100,101] suggested that the loss of polarity in the elongation of cylindrical cells might be due to the decomposition of some cytoplasmic components such as "microtubules" that normally control the direction of cell enlargement. Thus, it is quite interesting to see whether the change in cell shape is accompanied by any variation of the wall structure. In order to verify this point, microfibrillar orientation of parenchyma cell walls of colchicine-treated pine seedlings were examined[63]. As is shown in Figs. 37 and 38, two distinct microfibrillar orientations (one is oriented perpendicular as shown in Fig. 37 and the other oriented parallel as shown in Fig. 38 to the main cell axis) are observed in the innermost surface of the cell walls. The occurrence of longitudinally oriented microfibrils may not be the result of a change in microfibrillar orientation during cell swelling, but may be the result of longitudinal deposition itself. Taking into consideration that microtubules are destroyed by the colchicine treatment, the evidence that highly oriented microfibrils actually occur in the walls of swollen cells is incompatible with the suggestion of Green[100,101] mentioned above.

### 3.4 Cell swelling without reorientation of microfibrils

Looking at the multinet growth theory from another view that cells enlarge predominantly in a radial direction instead of a longitudinal one, the enlarging cells should have a restraining influence of axial elongation from newly deposited longitudinally oriented microfibrils. This view has been supported by Hogetsu *et al.*[98], Probine[93], Ridge[61], Sargent *et al.*[95,96], Shibaoka[97] and Veen[60] who demonstrated that the direction of newly deposited microfibrils changed from transverse to longitudinal by chemical reagents. The electron micrographs, however, showed mainly three types of microfibrillar orientation, one perpendicular, another oblique and the third parallel to the main cell axis. Hence, it is questionable that

coumarin and colchicine causes radial swelling of cells by changing microfibrillar orientation from perpendicular to parallel to the main cell axis.

Furthermore, HOGETSU *et al.*[98] and SHIBAOKA[97] reported that wall microtubules in epidermal cells of Azuki bean, enlarged by the treatment of kinetin together with IAA, ran parallel to the main cell axis, although transverse microtubules were observed in normal cells. They suggested that the change of microtubule orientation should necessarily be observed in radially enlarged cells prior to the change of microfibrillar orientation. On the other hand, wall microtubules in parenchyma cells of pine, enlarged by the treatment of coumarin, run parallel or perpendicular to the main cell axis. Epidermal walls of a variety of species are reported to have crossed polylamellate structures[55]. It seems possible that the longitudinally and transversely oriented microtubules should be found immediately under the plasmalemma in the epidermal cells of Azuki bean. Hence, longitudinal microtubules may not be caused by a microfibrillar change, but occur *in situ* without further change.

GREEN[100] noted that the meristematic cell of the filamentous alga, *Nitella*, when grown in the presence of colchicine, became spherical rather than cylindrical and that the cellulose microfibrils of the wall are randomly oriented. Furthermore, examination of colchicine-treated secondary walls of cultured stem segments of *Coleus* fixed in potassium permanganate[103] showed that the cellulose microfibrils lost their normal parallel orientation and were deposited in swirls and curved configurations. The electron micrographs of the recent study[63], however, showed that microfibrils deposited during the treatment with colchicine are not random but highly oriented. This fact suggests that the organization mechanism of highly oriented microfibrils may not be destroyed in colchicine-treated parenchyma cells even if colchicine has a direct degradative action on the microtubules as indicated by BORISY and TAYLOR[105]. Hence, synthesizing agents of cellulose microfibrils which may be situated in or near the plasmalemma and closely correlated with microtubules may not be influenced by colchicine, even if microtubules are destroyed. Microtubules may have a transient function in controlling microfibrillar orientation. This view is in good agreement with the suggestion of MARX-FIGINI[106,107] that microtubules are not involved in the biosynthesis of cellulose.

The present results that both transversely and longitudinally oriented microfibrils are frequently seen in the innermost surface of control, coumarintreated and colchicine-treated cells may conclude that cell swelling reagents do not have the property of changing microfibrillar orientation commonly.

Thus, orientation of microfibrils may not be changed from transverse to longitudinal in the walls of coumarin- and colchicine-treated cells, which again supports the "ordered fibril hypothesis" of ROLAND *et al.*[57].

## 4. Microfibrillar orientation of suspension-cultured cells

### 4.1 Preparation of suspension-cultured cells

In the preceding chapter, the wall structure having a polarity in cell elongation, such as tissue parenchyma cells, has been investigated. It was found that the cells have crossed polylamellate structure throughout the cell elongation. Furthermore, it was observed that the orientation of newly deposited microfibrils in the innermost wall surface is not only perpendicular but also oblique and parallel to the main cell axis.

According to the multinet growth hypothesis, the orientation of microfibrils formed on the innermost wall surface is usually perpendicular to the direction of cell elongation causing the restraint of the radial expansion of the cell. If a cell does not have polarity in its expansion, it is natural for this hypothesis to presume that the newly formed microfibrils always should have random orientation.

Recently, the suspension-cultured cells are increasingly utilized as experimental materials to investigate the formation of cell walls. Especially, many investigations have been performed to know the synthesis and orientation of cellulose microfibrils[108~115]. However, the organization of cell walls of the suspension-cultured cells is not studied yet in the ultrastructural level. Therefore, the present chapter deals with the wall structure of microfibrillar orientation of suspension-cultured cells, growing freely without polarity, which was investigated by the freeze etching

Table 3. LINSMAIER and SKOOG basal liquid medium.

| Mineral salts | | | |
|---|---|---|---|
| Major elements | | Minor elements | |
| Salts | mg/l | Salts | mg/l |
| $NH_4NO_3$ | 1,650 | $H_3BO_3$ | 6.2 |
| $KNO_3$ | 1,900 | $MnSO_4 \cdot 4H_2O$ | 22.3 |
| $CaCl_2 \cdot 2H_2O$ | 440 | $ZnSO_4 \cdot 4H_2O$ | 8.6 |
| $MgSO_4 \cdot 7H_2O$ | 370 | KI | 0.83 |
| $Na_2EDTA$ | 37.3 | $CuSO_4 \cdot 5H_2O$ | 0.025 |
| $FeSO_4 \cdot 7H_2O$ | 27.8 | $CoCl_2 \cdot 6H_2O$ | 0.025 |
| Organic constituents | | | |
| Sucrose | 30 g/l | Thiamine•HCl | 0.4 mg/l |
| | | myo-Inositol | 100 mg/l |
| Hormones | | | |
| 2, 4-D | $10^{-6}$ M | Kinetin | $10^{-6}$ M |

* pH is adjusted to 5.6 by 0.2 N NaOH.

technique without pretreatment.

Callus cells induced from tobacco tissue (*Nicotiana tabacum* L.) at the laboratory of Plant Nutrition in the Department of Agricultural Chemistry, Kyoto University in 1968 on LINSMAIER and SKOOG basal medium[116] with $10^{-5}$ M 2, 4 dichlorophenoxy-acetic acid (2, 4-D) were offered to use. The callus cells in LINSMAIER and SKOOG basal liquid medium (Table 3) (with $10^{-6}$ M 2, 4-D) were subjected to continuous reciprocal shaking culture in the dark. Callus of *Rauwolfia serpentina* Benth stems had been induced with $10^{-5}$ M 2, 4-D in 1970, and continuously cultured in a modified LINSMAIER and SKOOG basal medium (with $10^{-6}$ M 2, 4-D) in the same laboratory. The callus cells in the modified LINSMAIER and SKOOG basal liquid medium (with $10^{-6}$ M 2, 4-D)[117] were also subjected to continuous reciprocal shaking culture in the dark.

Actively growing suspension cells of both *Nicotiana* and *Rauwolfia* of 4 to 5 days after resuspension of the cells were transferred to test tubes respectively. After short leave, the supernatant was transferred to a centrifugi tube with a pipette and centrifuged. The precipitates of cultured cells were put on a specimen holder as rapidly as possible and immediately frozen by immersion in Freon 12 maintained nearly at its freezing point. Then, the samples were treated by the same way as described in the preceding paper[25].

### 4.2 Microfibrillar orientation observed by the freeze etching technique without pretreatment

It is indicated that the deep etching modification of the freeze etching technique enables the nature of the substances formed beneath the polylamellate wall to be clearly defined[38,39].

The present technique of freeze fracturing alone without further etching, however, revealed the detailed structure of quite a thin polylamellate wall and the relationship between plasmalemma and the newly synthesized cellulose microfibrils.

Cytoplasmic details of the cells pretreated with glutaraldehyde and glycerol solution were well preserved without the damage of ice crystals. The plasmalemma surface was characterized by the presence of numerous plasmalemma particles and numerous protrusions. On the other hand, cytoplasm of the cells without any pretreatment was also well preserved without ice formation. This was presumed that sucrose added in the culture medium in the concentration of 3%(w/w) raised the sugar concentration in the cytoplasm of cultured cells and acted as a cryo-protective agent just the same as glycerol.

Furthermore, the sculpture on the plasmalemma without pretreatment was conspicuous in that the fractured plane of the plasmalemma was characterized by the imprint of microfibrils of the innermost lamella as narrow grooves (on the

P-face of plasmalemma) or as protruded lines (on the E-face of plasmalemma) (Figs. 39 and 40).

A similar phenomenon is already described in the case of cortical parenchyma cells of poplar (see Chapter 1).

Therefore, it was further strengthened that the imprint of microfibrils on the fractured plane of plasmalemma was observed only by the freeze fracturing(or freeze etching) technique without pretreatment.

Although some imprints of microfibrils of both *Nicotiana* and *Rauwolfia* were oriented in random fashion, the others of them were oriented regularly in the same direction in respective lamellae (Figs. 39 and 40). Fig. 40 shows two types of orientation of the imprint of microfibrils; one is indicated by a long arrow which corresponds to the direction of microfibrils in the innermost lamella and the other is indicated by a short arrow which corresponds to the direction of microfibrils of the lamella immediately outside the innermost lamella. Thus, the imprints of microfibrils were crisscrossed between each lamella, and that the orientation of microfibrils was observed through the portion of torn plasmalemma (Fig. 41). The figure shows microfibrils of the innermost surface of the wall of *Rauwolfia* exposed by the tearing-off of the plasmalemma. Much clear crossed lamellate structure which consisted of two types of microfibrillar orientation is also shown in this figure.

The occurrence of crossed lamellate structure was not the exception in the case of suspension-cultured cells of *Nicotiana* (Fig. 42).

Throughout these observations, it was supposed that the cell walls of higher plants were controlled fundamentally to keep regular patterns of microfibrils, because microfibrils were oriented not randomly but regularly even in suspension-cultured cells which have no polarity in cell expansion.

On the basis of the above results, the occurrence of crossed polylamellate structure of the walls of tissue parenchyma cells may not be the results of passive reorientation of microfibrils as assumed by the multinet growth hypothesis, but the results of inherent nature of the cells.

## Conclusion

The structure and growth of primary walls of some woody plant cells were studied mainly by the freeze etching technique.

The most characteristic feature on the plasmalemma surface of poplar parenchyma cells observed by the technique is the occurrence of "fibrillar structures". The "fibrillar structures" which are observed by the freeze etching only after fixation and glycerol impregnation are considered to be imprint of underlying microtubules on the plasmalemma surface. Furthermore, the technique seemed to be highly

reliable and suitable for the study of natural states of microfibrillar orientation and its organization, because it gives highly oriented microfibrils in any lamella and enables to do very good insight into three dimensional relationship among cytoplasm, plasmalemma and cell wall. The freeze etching without any pretreatment shows the impressions of ordered and compact microfibrils on the plasmalemma, so that it is a quite useful technique for the investigation of the more intact orientation of just deposited microfibrils and of the function of the plasmalemma which may be involved in the orientation and synthesis of microfibrils.

Based on the above availability of the freeze etching technique, the following informations were obtained.

1. The parenchyma cell walls in the primary tissue of three angiosperms, namely, *Populus nigra* L. var. *italica* Koehne, *Morus bombysis* Koidz. and *Phaseolus vulgaris* var. *humilis* Alef. had a crossed polylamellate structure.

2. Three types of microfibrillar orientation, namely, parallel, perpendicular and oblique to the main cell axis were found not only on the innermost surface but also throughout the developing poplar parenchyma cell wall. Each set of microfibrils was crossed with the underlying microfibrils. Furthermore, three types of microtubule orientation, namely, parallel, perpendicular and oblique to the main cell axis were observed, coinciding with those of microfibrils.

3. The walls of cortical parenchyma cells (third or fourth cell inward from epidermis) of poplar showed an increase of wall thikness or wall thickening during elongation growth, and also showed crossed polylamellate structures in almost all the stages of cell elongation.

4. Three main types of microfibrillar orientation were also observed on the innermost surface of the walls of control, coumarin-treated and colchicine-treated radially swollen cells of pine seedlings; the first was parallel, the second was perpendicular and the third was oblique to the main cell axis. The results indicate that the cell swelling reagents do not have the property of changing microfibrillar orientation.

5. Suspension-cultured cells of *Rauwolfia* and *Nicotiana* without polarity in cell expansion have highly oriented microfibrils and sometimes crossed lamellate structures.

In conclusion, these findings lead to the suggestion that cortical parenchyma cells actively change the direction of depositing microfibrils in any stages of their elongation by determining the orientation of microfibrils at the time of their deposition such as perpendicular, oblique or parallel to the main cell axis and totally constitute the crossed polylamellate structure in their walls. These results are well explained by the modified "oredered fibril hypothesis" presented newly in this study.

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## Explanation of figures

Abbreviations used:

CW: cell wall
PL-E: E-face of plasmalemma
PPF: primary pit field
V: vacuole
↔: direction of the main cell axis
F: "fibrillar structure"
PL-P: P-face of plasmalemma
T-P: P-face of tonoplast
⟲: direction of shadowing

Fig. 1. Freeze fractured plasmalemma of a *Populus* parenchyma cell. Fibrillar structures are shown as long concave striations on the E-face of the plasmalemma. On the P-face of the plasmalemma, they are shown as long convex striations. ×10,000.

Fig. 2. Freeze fractured plasmalemma of a *Populus* parenchyma cell. Fractured plane of the tonoplast and plasmalemma observed from the cytoplasmic side. Some fibrillar structures pass from the surface of the tonoplast (convex striations; short arrows) to that of the plasmalemma (concave striations; long arrows). ×10,000.

Fig. 3. Freeze fractured E-face of plasmalemma of a *Populus* cortical parenchyma cell without pretreatment. Protruded lines, which are highly oriented, indicate the impressions of microfibrils. ×21,000.

Fig. 4. Freeze fractured plasmalemma of a *Populus* parenchyma cell without pretreatment. P-face of plasmalemma is shown. The impressions of microfibrils are seen as narrow grooves running orderly. ×20,000.

Fig. 5. Freeze fractured plasmalemma of a *Populus* parenchyma cell without pretreatment. Arrows indicate the wall microfibrils exposed by tearing-off of plasmalemma. ×21,000.

Fig. 6. Freeze dried wall of a *Populus* parenchyma cell. Microfibrils in the innermost surface run perpendicular to the main cell axis. ×21,000.

Fig. 7. Freeze etched wall of a *Populus* parenchyma cell. Microfibrils in the exposed surface of a fractured wall run perpendicular to the main cell axis. Microfibrils of the overlying lamella (short arrows) run oblique to the main cell axis. ×14,000.

Fig. 8. Freeze dried wall of a *Populus* parenchyma cell. Microfibrillar orientation in the innermost surface is parallel to the main cell axis. The underlying microfibrils are oriented almost perpendicular to the main cell axis. ×6,500.

Fig. 9. Freeze etched wall of a *Populus* parenchyma cell. Microfibrils run almost parallel to the main cell axis. The underlying microfibrils run almost perpendicular to the main cell axis. ×12,000.

Fig. 10. Freeze dried wall of a *Populus* parenchyma cell. Microfibrils in the innermost surface run oblique to the main cell axis. Microfibrils of the surface lamella are crossed with those of the underlying lamella. ×21,000.

Fig. 11. Freeze fractured wall of a *Populus* parenchyma cell. Transverse, oblique and longitudinal microfibrils are seen. ×13,000.

Fig. 12. Freeze fractured wall of a *Populus* parenchyma cell. Oblique microfibrils which show a separation longitudinally are seen. ×13,000.

Fig. 13. Freeze etched wall of a *Populus* parenchyma cell. Microfibrils in the exposed surface run oblique to the main cell axis. The underlying microfibrils, the direction of which is shown by short arrows, take a reverse orientation which is seen at primary pit fields. ×16,000.

Fig. 14. Freeze fractured plasmalemma of a *Populus* parenchyma cell without pretreatment. The imprints of the innermost wall microfibrils run perpendicular to the main cell axis. ×7,000.

Fig. 15. Freeze fractured plasmalemma of a *Populus* parenchyma cell without pretreatment. The imprints of the innermost wall microfibrils run oblique to the main cell axis. ×13,000.

Fig. 16. Freeze fractured plasmalemma of a *Populus* parenchyma cell without pretreatment. The imprints of the innermost wall microfibrils run parallel to the main cell axis. ×13,000.

Fig. 17. Freeze fractured plasmalemma of a *Populus* parenchyma cell without pretreatment. The imprints of the innermost wall microfibrils run oblique, while those of the underlying microfibrils run perpendicular to the main cell axis. ×21,000.

Fig. 18. Freeze etched plasmalemma of a *Populus* parenchyma cell. Transversely oriented "fibrillar structures" (short arrows) are shown. ×7,000.

Fig. 19. Freeze etched plpsmalemma of a *Populus* parenchyma cell. Longitudinally oriented "fibrillar structures" (short arrows) are shown. ×7,000.

Fig. 20. Freeze etched plasmalemma of a *Populus* parenchyma cell. Obliquely oriented fibrillar structures (short arrows) are shown. ×7,000.

Fig. 21. Ultra-thin section of *Populus* parenchyma cell wall shadow-casted with metals after removal of embedding materials. The area in which three single walls ($W_1$, $W_2$, $W_3$)

are conjugated was cut nearly parallel to the main cell axis. Crossed microfibrillar structure (Mi) is shown in $W_2$. ×18,000.

Fig. 22. Ultra-thin section of *Populus* parenchyma cell wall shadow-casted with metals after removal of embedding materials. A double cell wall cut almost parallel to the main cell axis is shown. The crossed polylamellate structure is quite distinct in Wb. ×18,000.

Fig. 23 and 24. Ultra-thin section of *Populus* parenchyma cell walls shadow-casted with metals after removal of embedding materials. Crossed polylamellate structures are shown in the parenchyma cells at 20 mm (Fig. 23) and 30 mm (Fig. 24) from the shoot apex. ×7,000 (Fig. 23) and ×7,000 (Fig. 24).

Fig. 25. Ultra-thin section of a *Populus* parenchyma cell wall shadow-casted with metals after removal of embedding materials. The section is sliced obliquely to the longitudinal axis. Crossed polylamellate structure is shown throughout the wall except the outermost part which has longitudinal microfibrils. ×18,000.

Fig. 26. Freeze fractured longitudinal wall of a *Populus* parenchyma cell, which was found to have a definite crossed lamellate structure; nearly transversely oriented microfibrils (long arrows) in these lamellae are alternated with longitudinally oriented ones (short arrows). ×10,000.

Fig. 27. Freeze fractured wall and plasmalemma surface of a *Phaseolus* parenchyma cell are shown. Some primary pit fields can be seen on the plasmalemma surface. ×2,500.

Fig. 28. High magnification image of the inlet of Fig. 27. Lamellae in which microfibrils are oriented perpendicular to the longitudinal cell axis (short arrows) are alternated with lamellae having a 40° angle to the longitudinal cell axis (long arrows). ×21,000.

Fig. 29. Freeze etched wall of a *Morus* parenchyma cell, in which crossed polylamellate structure is shown. The fibrillar crossing angle between any two adjacent lamellae is 40°. ×19,000.

Fig. 30. Freeze etched wall of a *Populus* parenchyma cell. Crossed polylamellate structure of the wall is shown. Microfibrils are oriented in two directions as indicated by short arrows. ×22,000.

Fig. 31. Direct carbon replica of a freeze-dried wall of a non-treated cortical parenchyma cell of *Pinus*. Microfibrils run parallel to the main cell axis, although some non-parallel microfibrils could be seen. ×21,000.

Fig. 32. Direct carbon replica of a freeze-dried wall of a coumarin-treated cortical parenchyma cell of *Pinus*. The orientation of microfibrils in the innermost lamella is parallel to the main cell axis. Microfibrils of the lamella immediately under the surface lamella run perpendicular to the main cell axis. ×22,000.

Fig. 33. Ultra-thin cross section of a coumarin-treated cortical parenchyma cell of *Pinus*. Microtubules (arrows) run parallel to the main cell axis which is perpendicular to the photographic plane. ×33,000.

Fig. 34. Direct carbon replica of a freeze-dried wall of a coumarin-treated cortical parenchyma cell of *Pinus*. The orientation of microfibrils in the innermost lamella is almost perpendicular to the main cell axis. ×25,000.

Fig. 35. Ultra-thin cross section of a coumarin-treated cortical parenchyma cell of *Pinus*. Microtubules (arrows) run perpendicular to the main cell axis which is perpendicular to the photographic plane. ×27,500.

Fig. 36. Direct carbon replica of a freeze-dried wall of a coumarin-treated cortical parenchyma cell of *Pinus*. Microfibrils cross the main cell axis diagonally. ×25,000.

Fig. 37. Direct carbon replica of a freeze-dried wall of a colchicine-treated cortical parenchyma cell of *Pinus*. Microfibrils run almost perpendicular to the main cell axis. ×21,000.

Fig. 38. Direct carbon replica of a freeze-dried wall of a colchicine-treated cortical parenchyma cell of *Pinus*. Microtubules run almost parallel to the main cell axis. ×21,000.

Fig. 39. Freeze fractured plasmalemma of a suspension-cultured cell of *Nicotiana* without pretreatment. The imprint of microfibrils run in regular pattern. ×13,000.

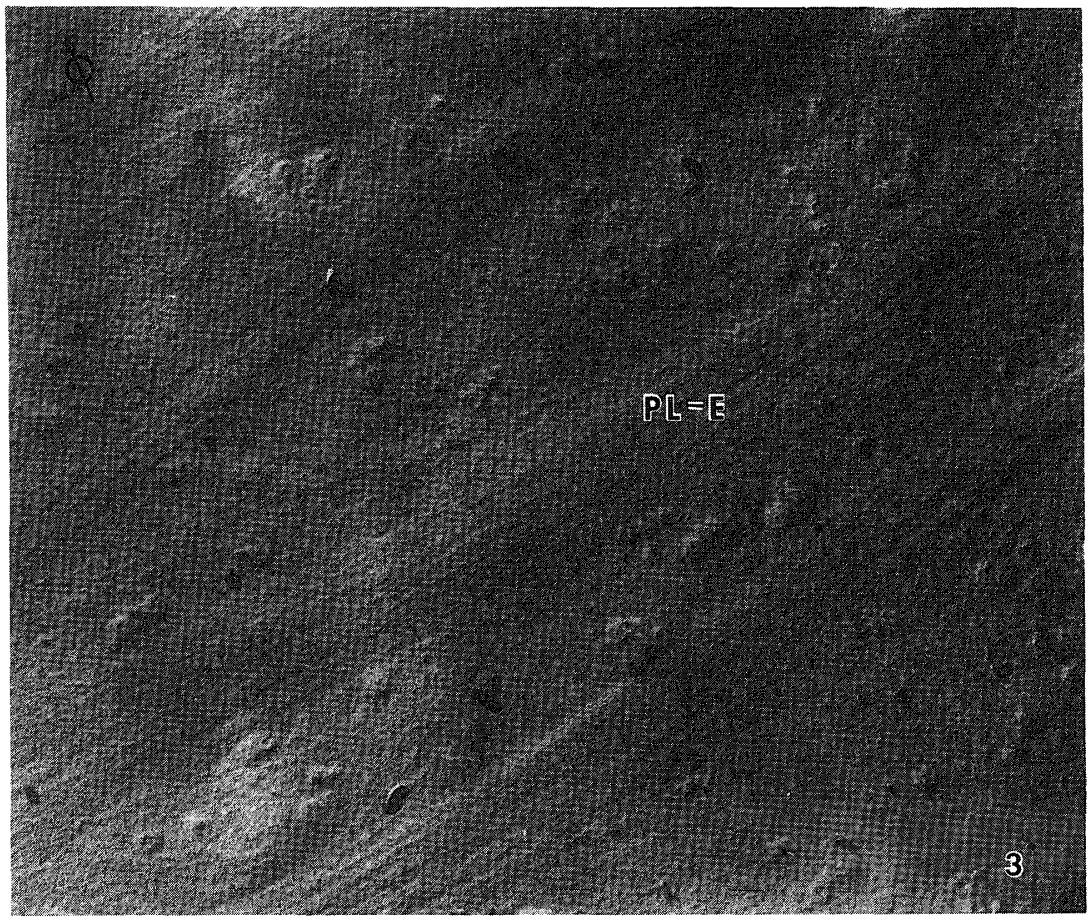
Fig. 40. Freeze fractured cytoplasm and plasmalemma of a suspension-cultured cell of *Rauwolfia*. Cytoplasmic organelles are well preserved. The imprint of microfibrils orient regularly as shown by arrows indicating crossed microfibrillar structure. ×13,000.

Fig. 41. Freeze fractured plasmalemma of a suspension-cultured cell of *Rauwolfia*. Microfibrils, exposed by tearing-off of plasmalemma, orient in two directions as shown by arrows. ×32,000.

Fig. 42. Freeze fractured wall of a suspension-cultured cell of *Nicotiana*. Oriented microfibrils are seen in crossed fashion. ×13,000.


C.W
F
F
P.L-P
P.L-E
F
1


C-W
F
PL-E
F
F
T-P
V
2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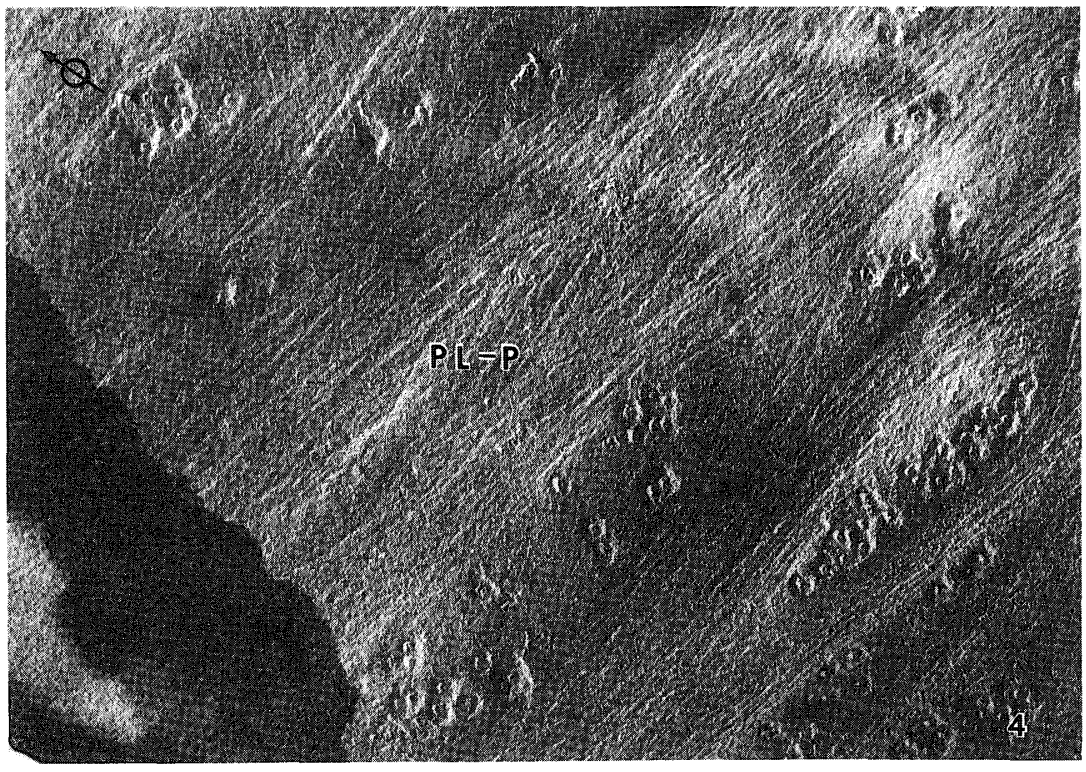
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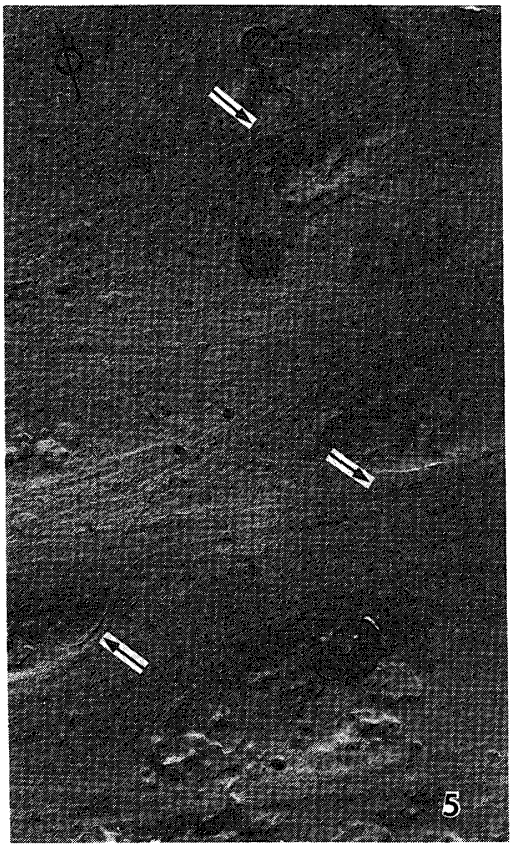

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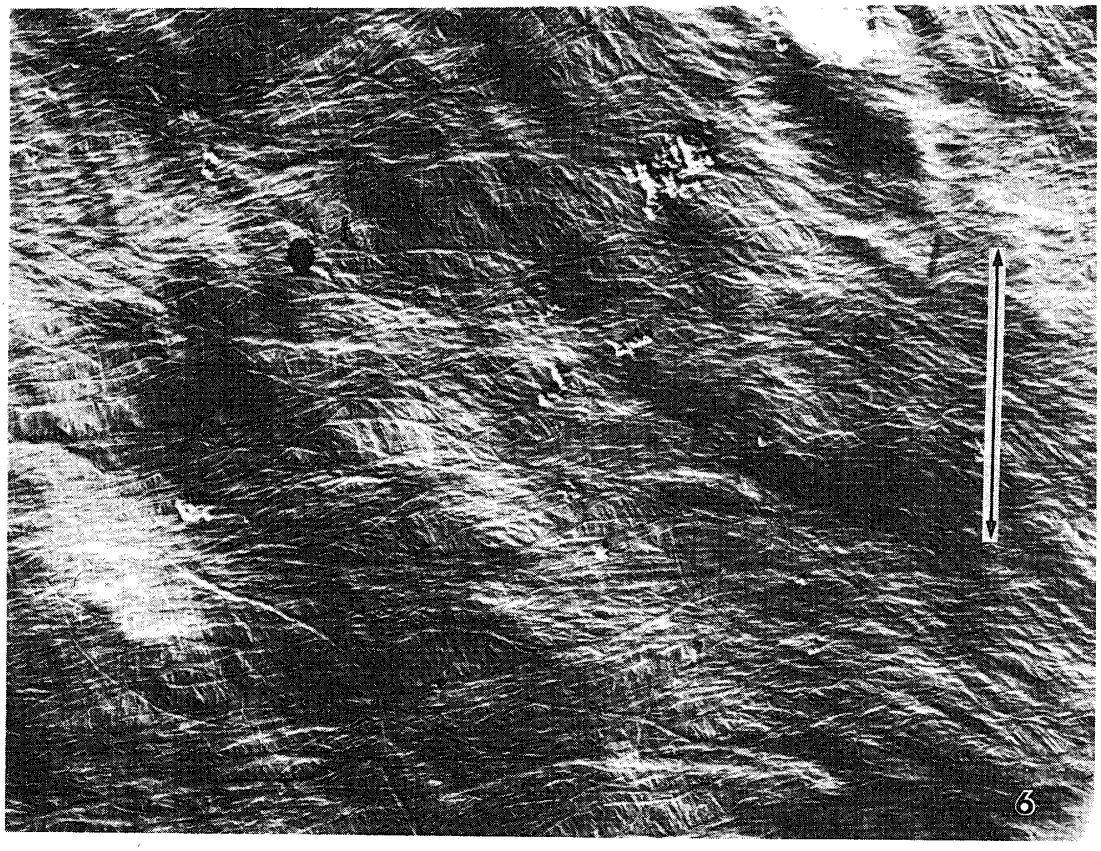

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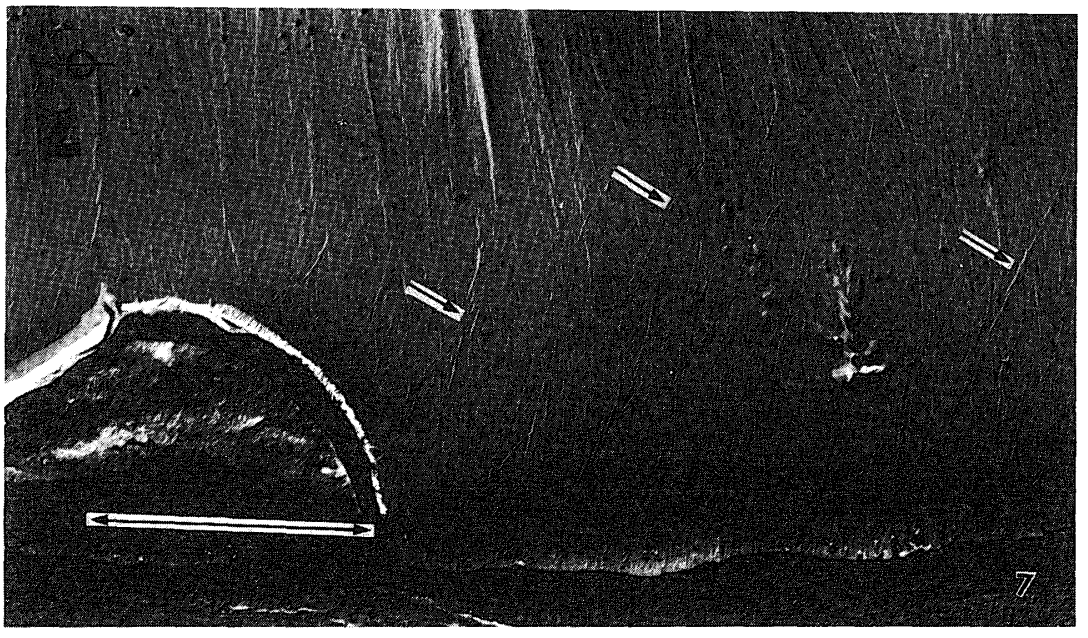
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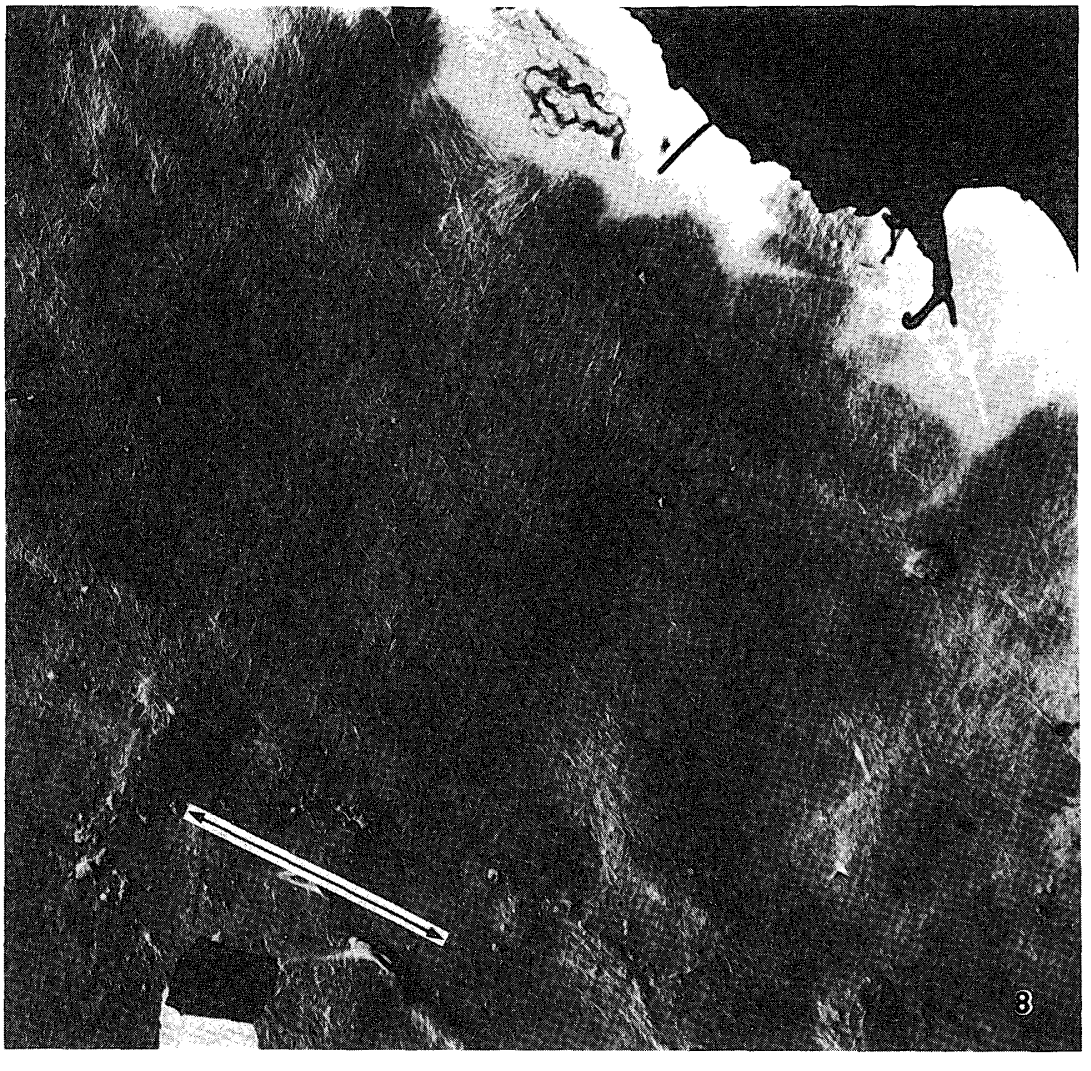
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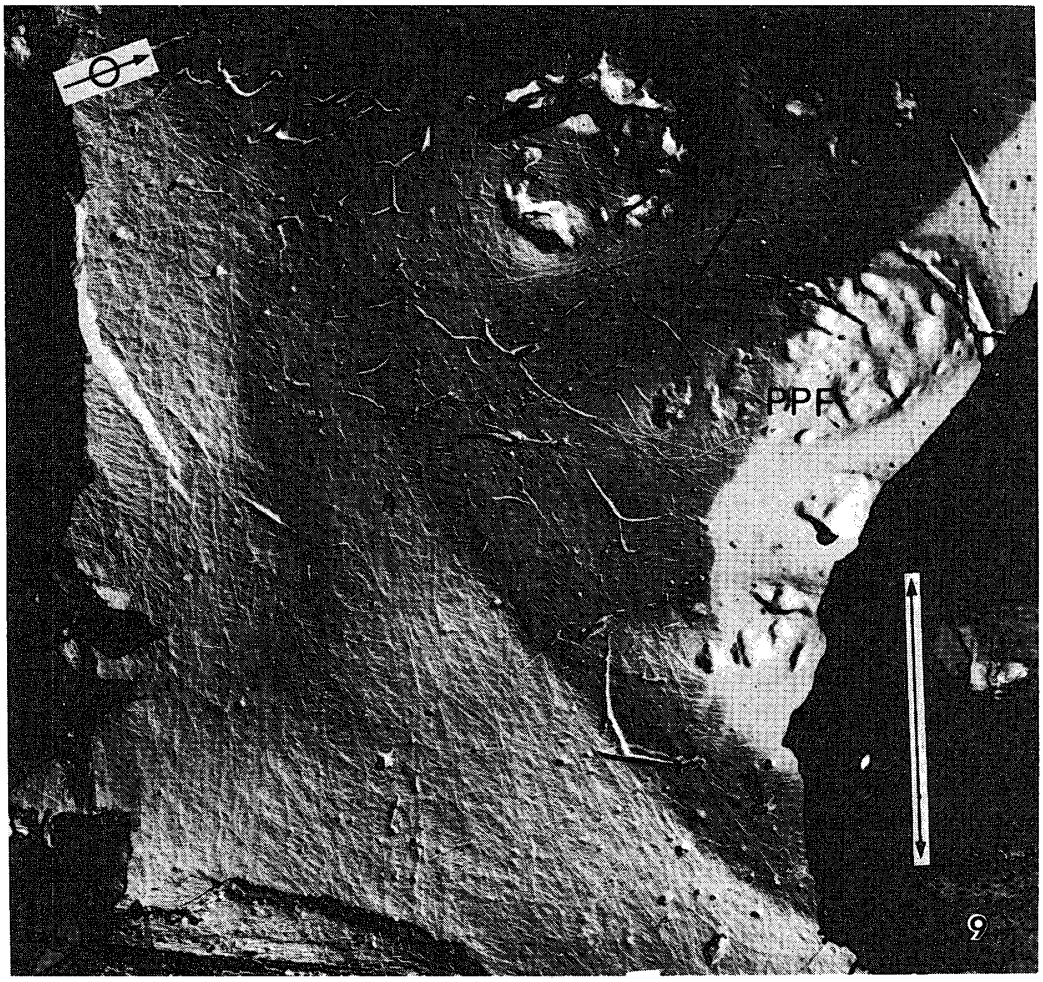
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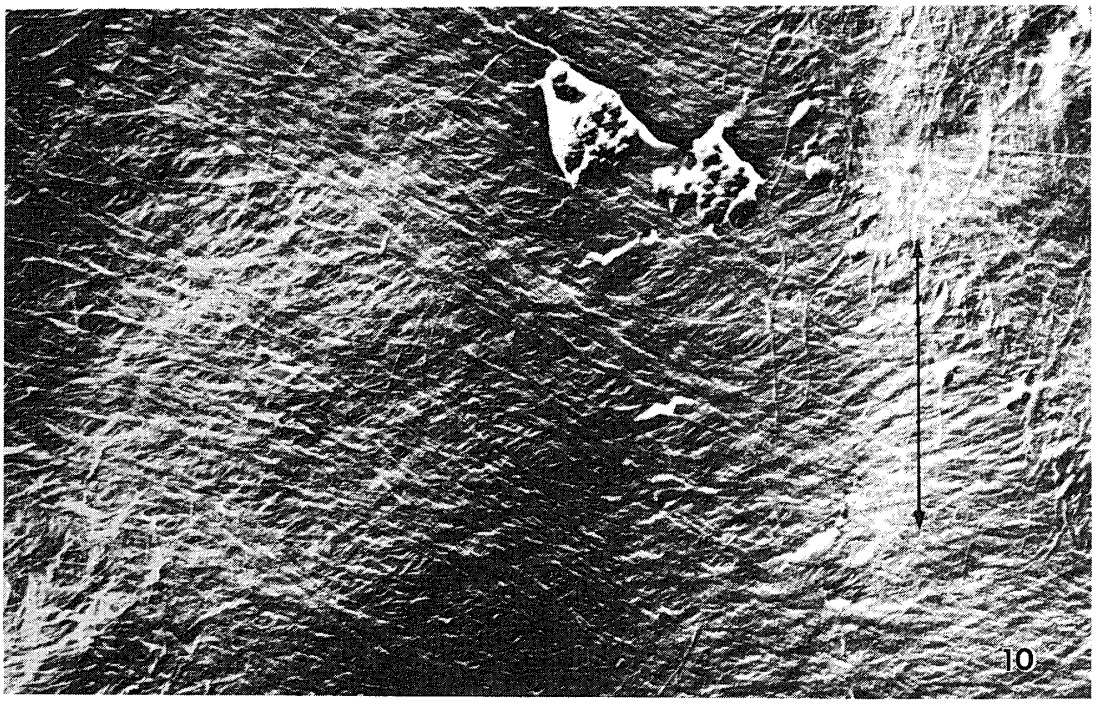
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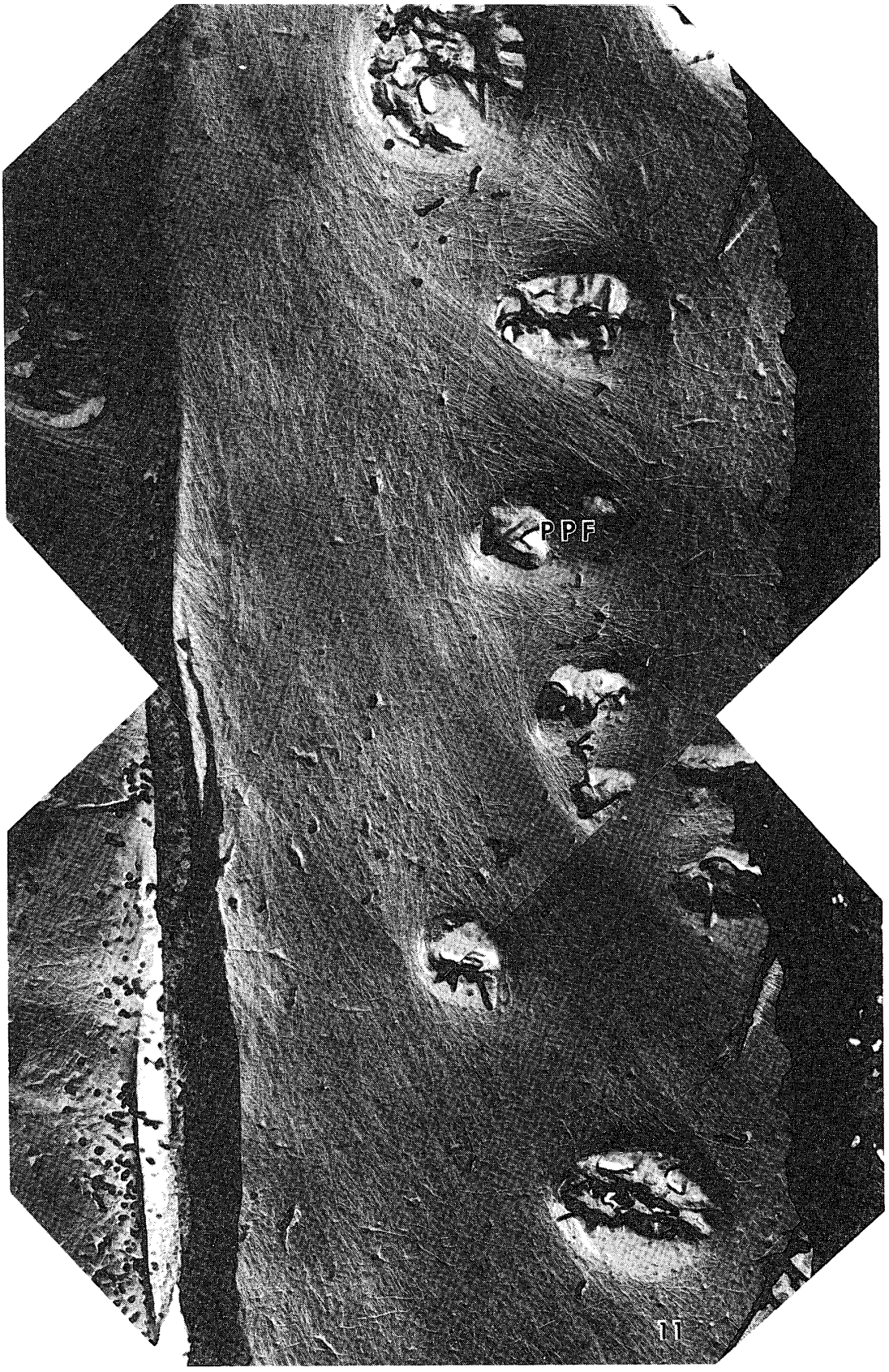
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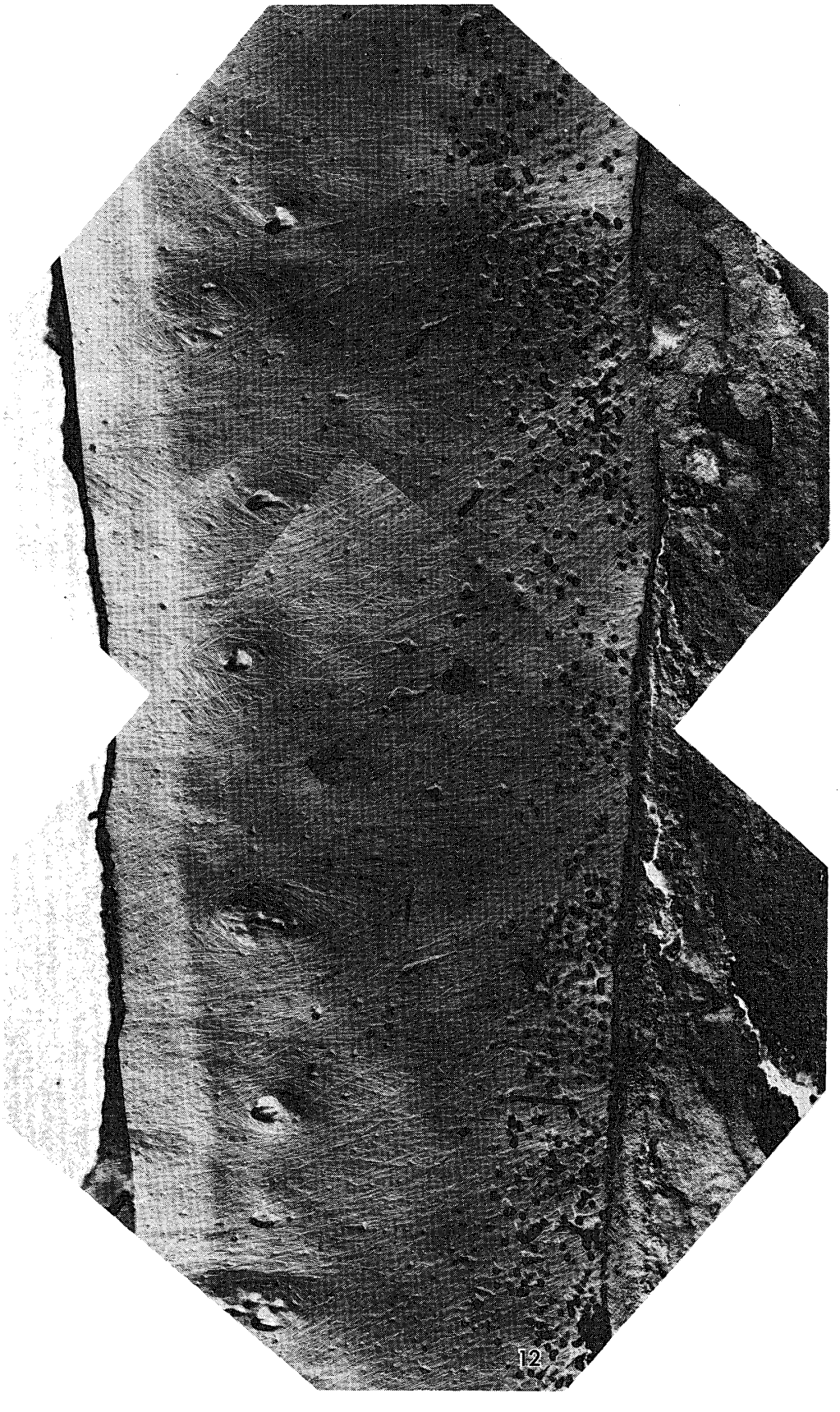
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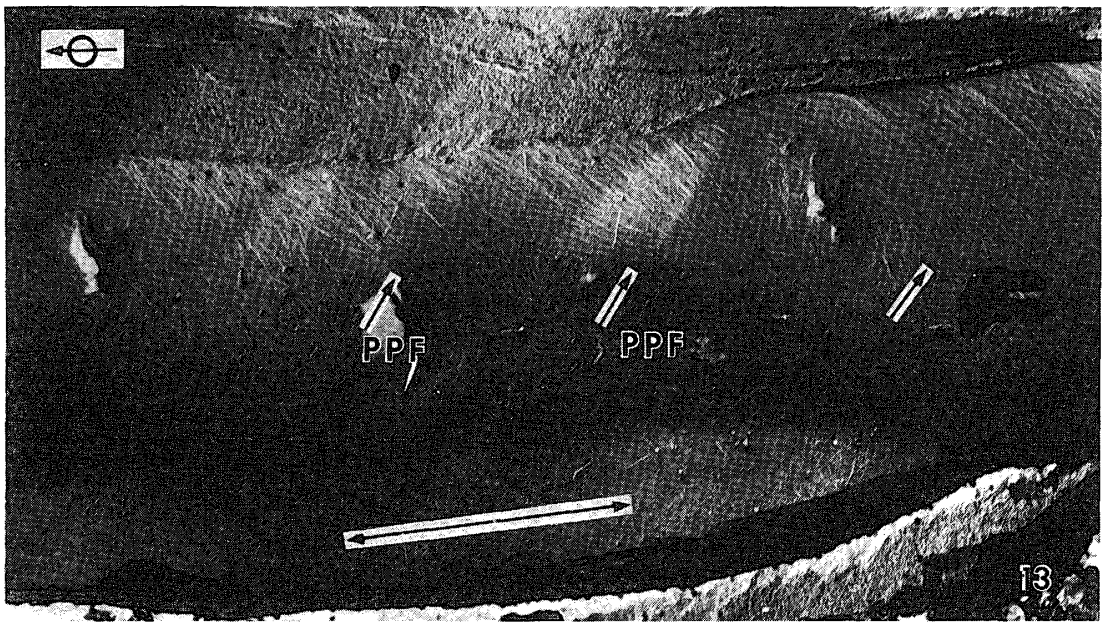
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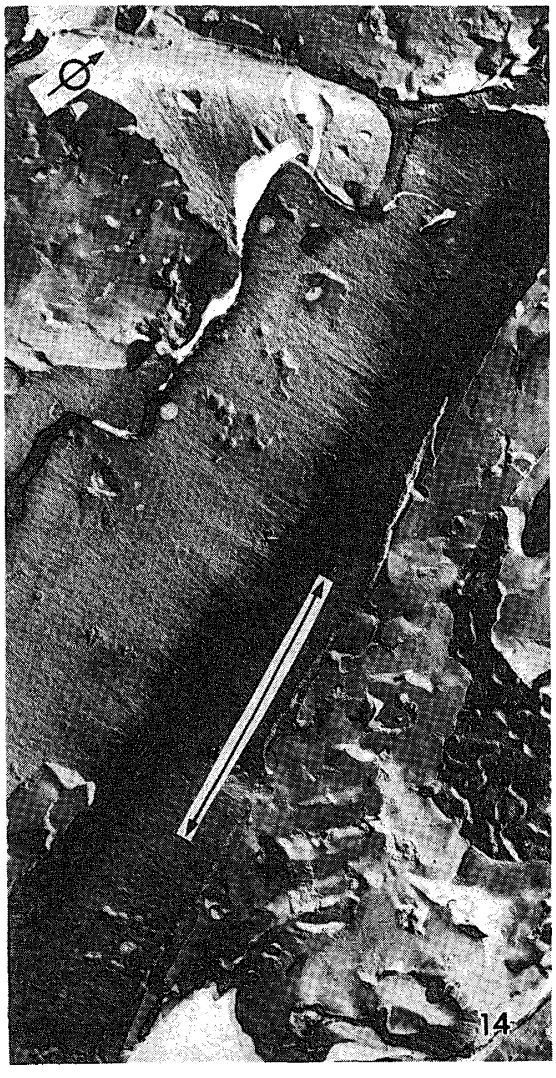
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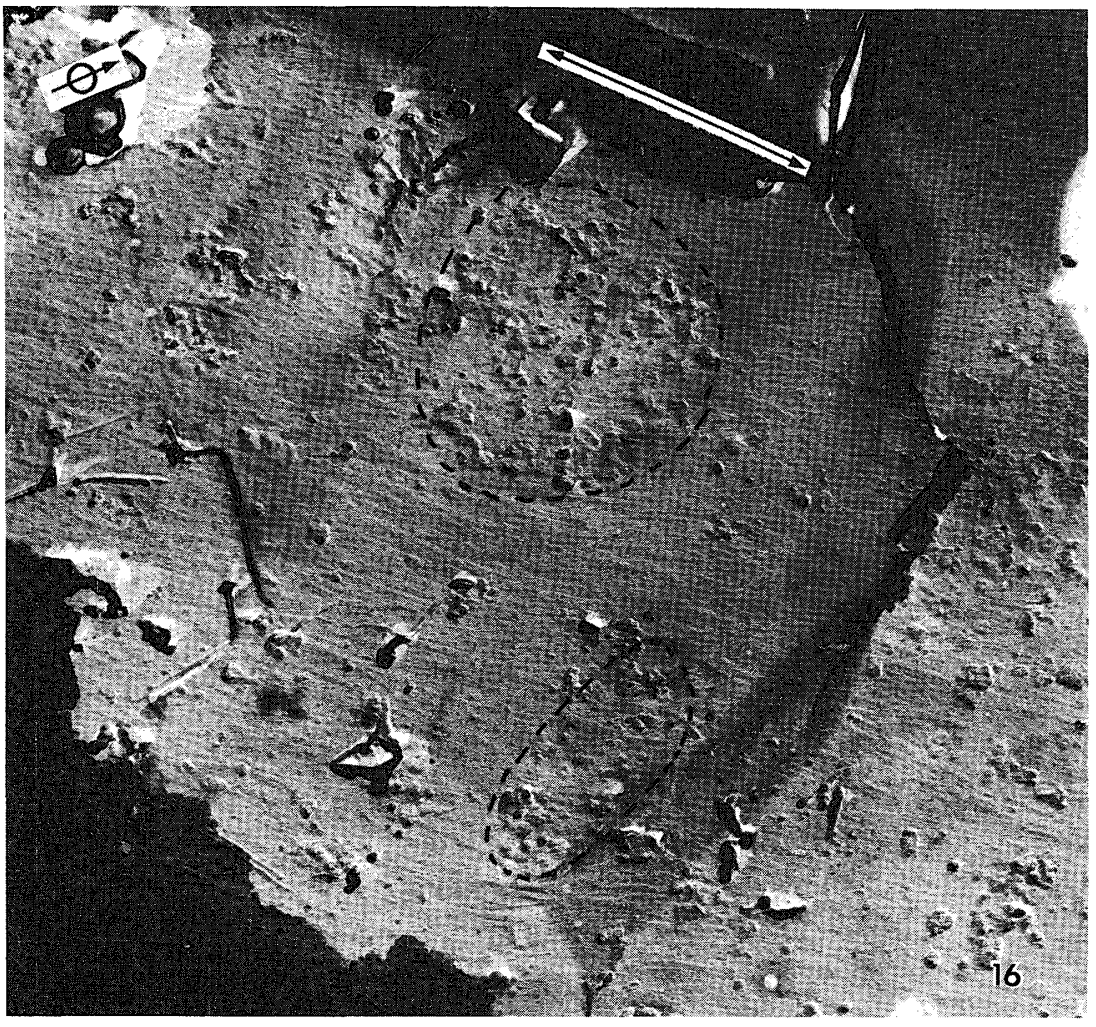
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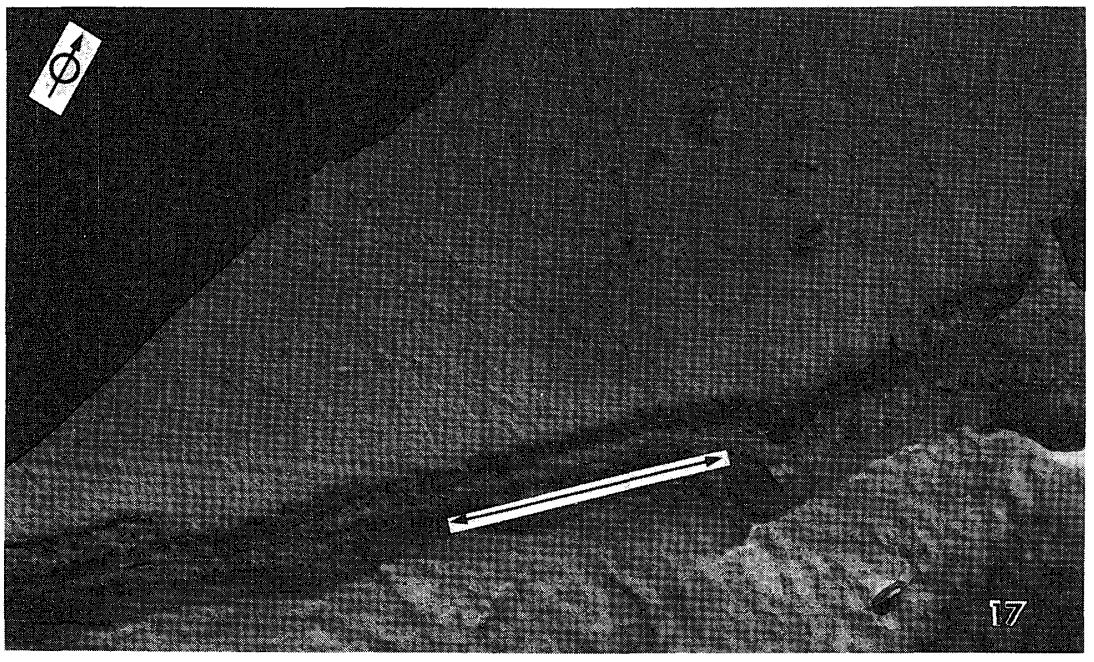
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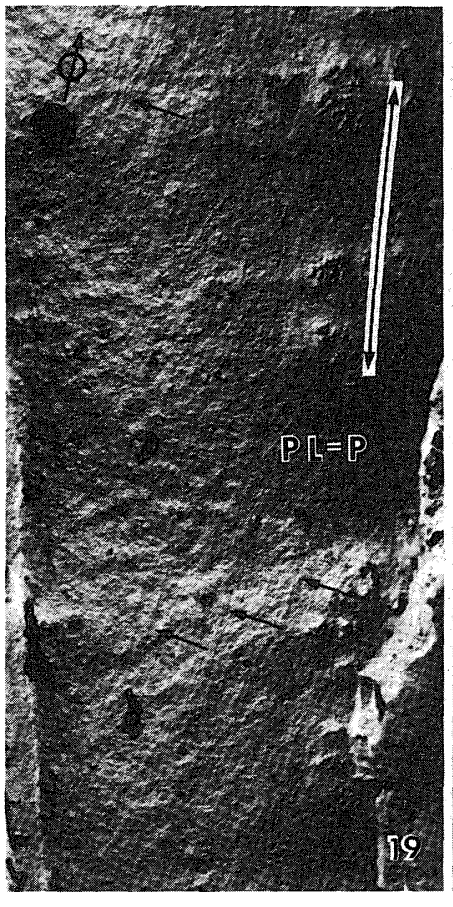
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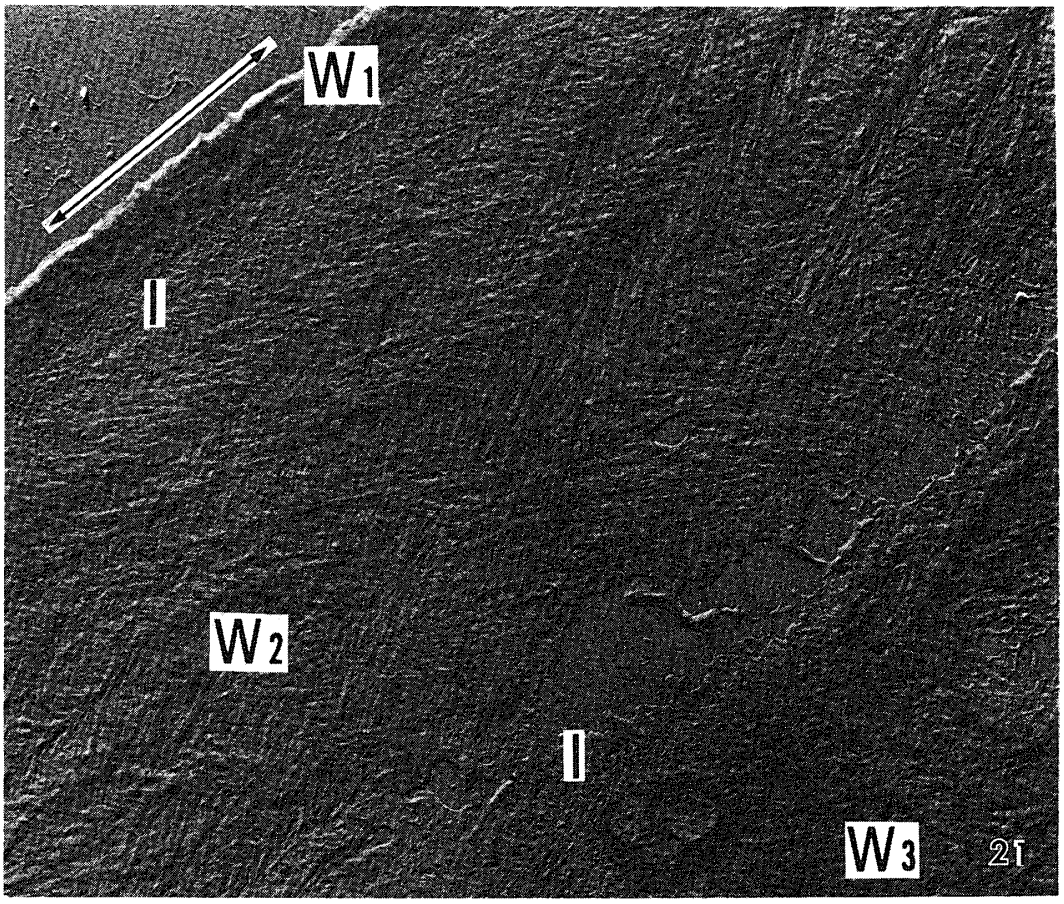
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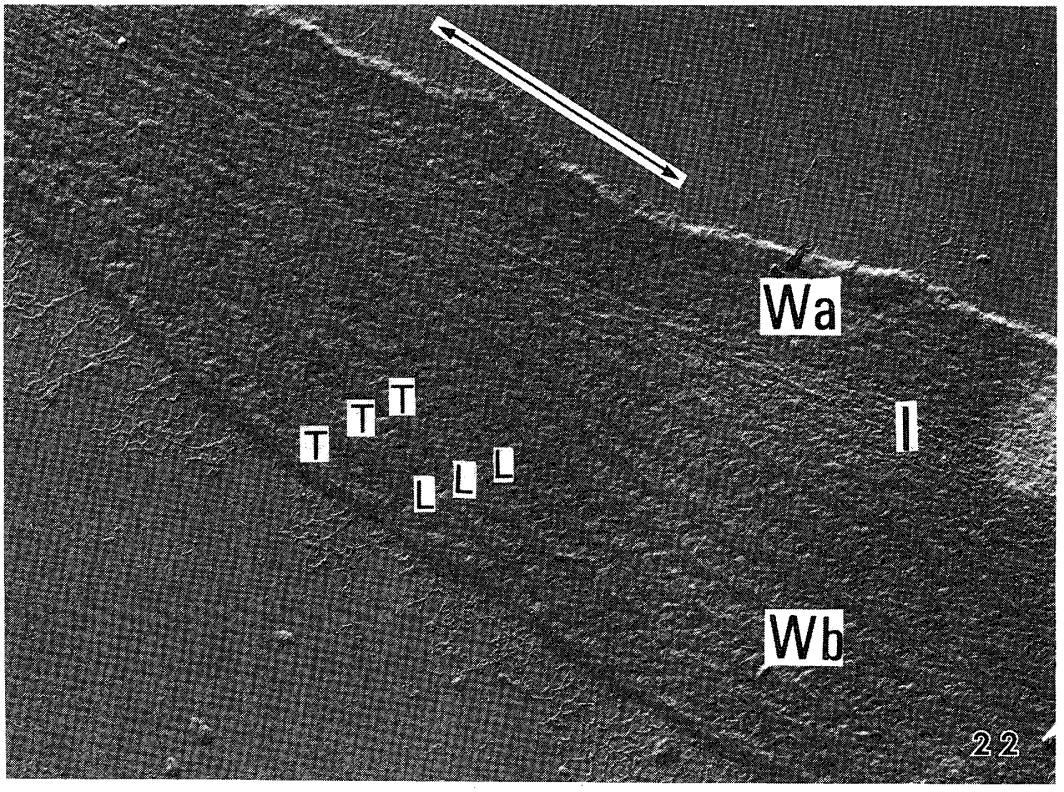
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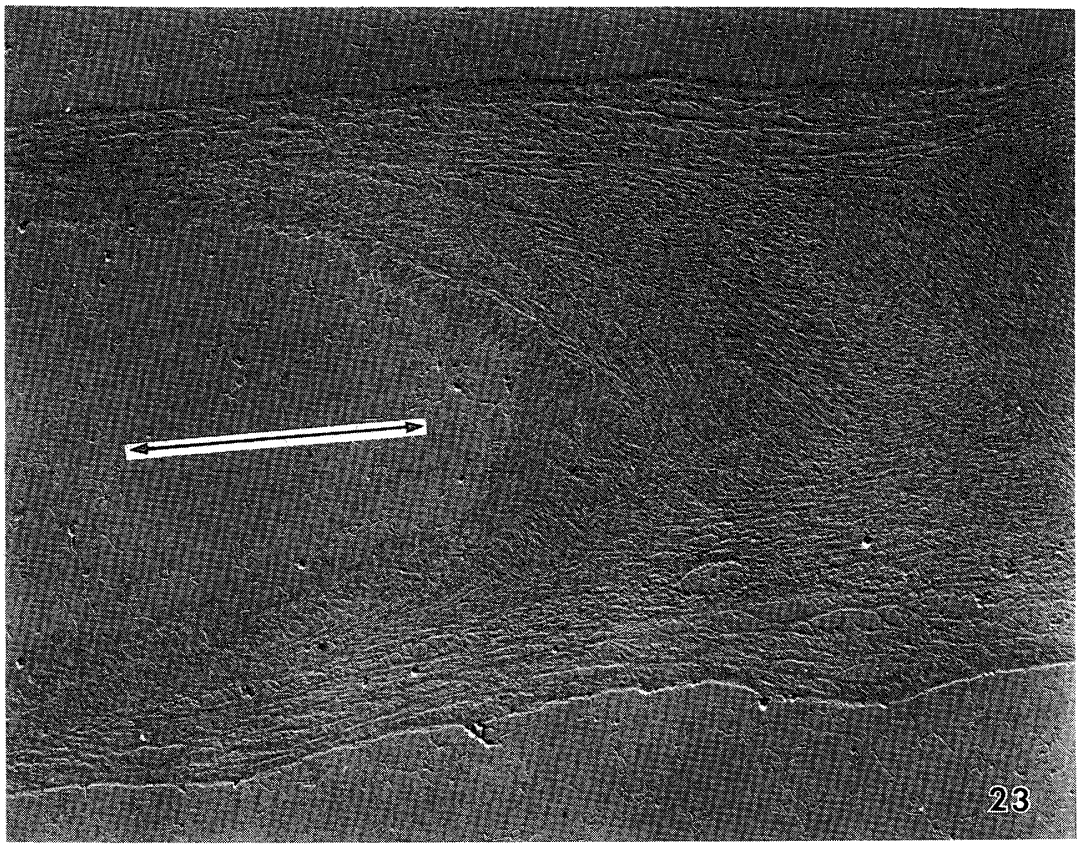
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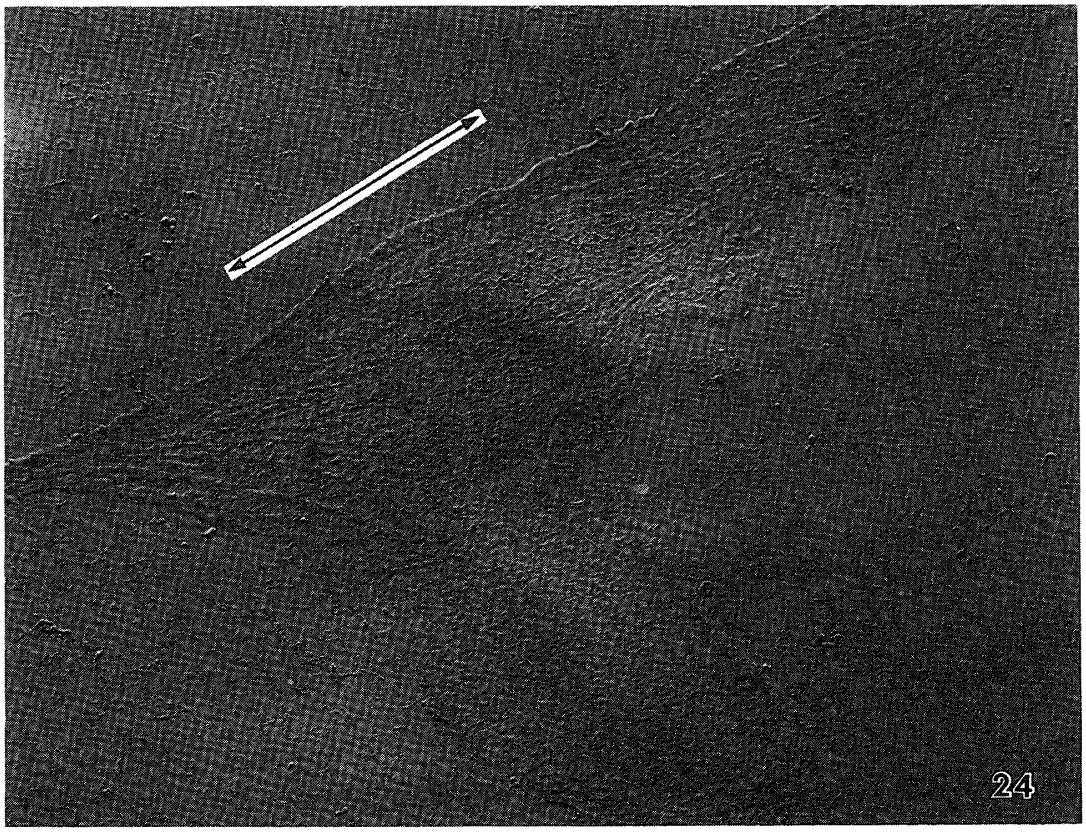
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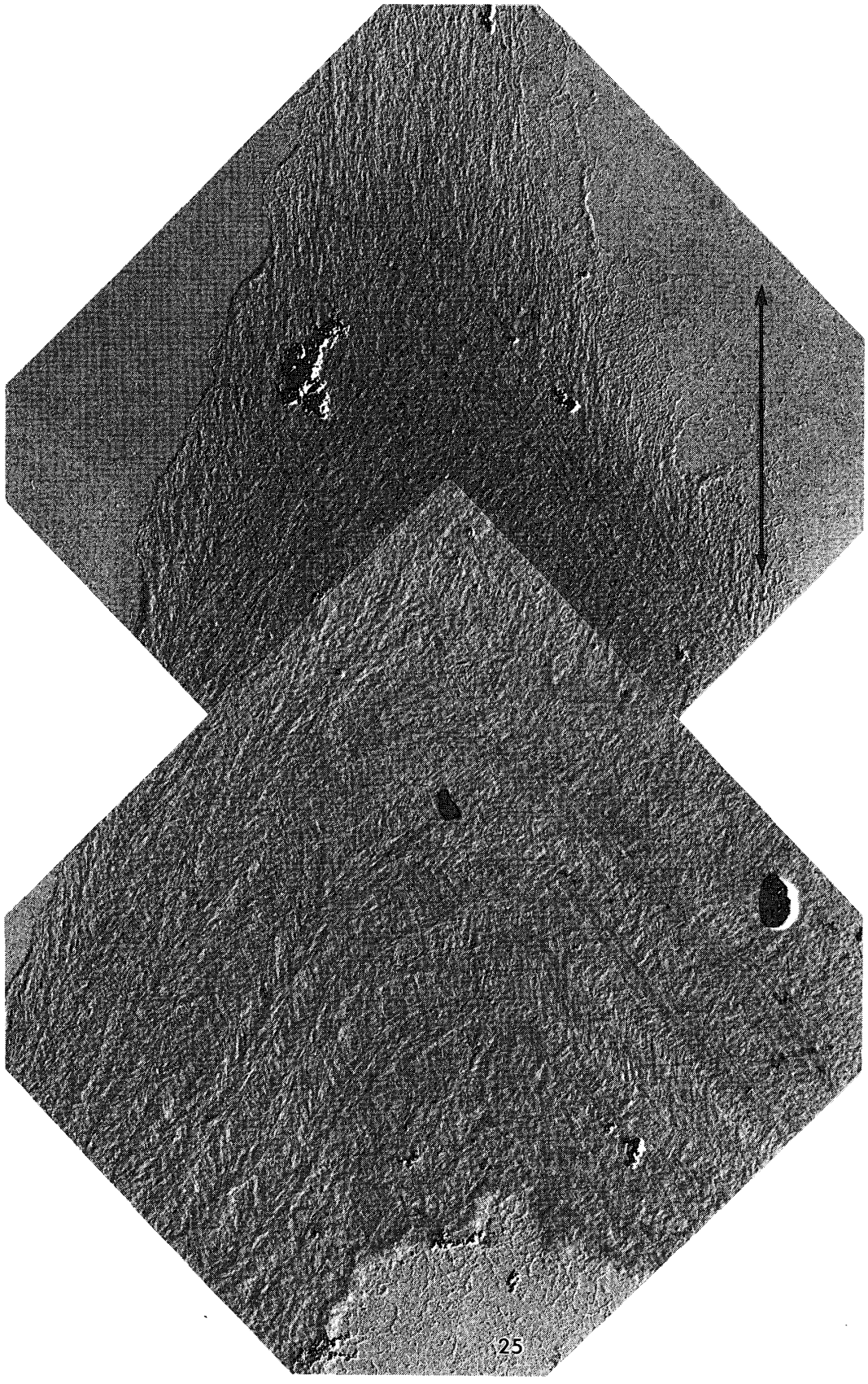
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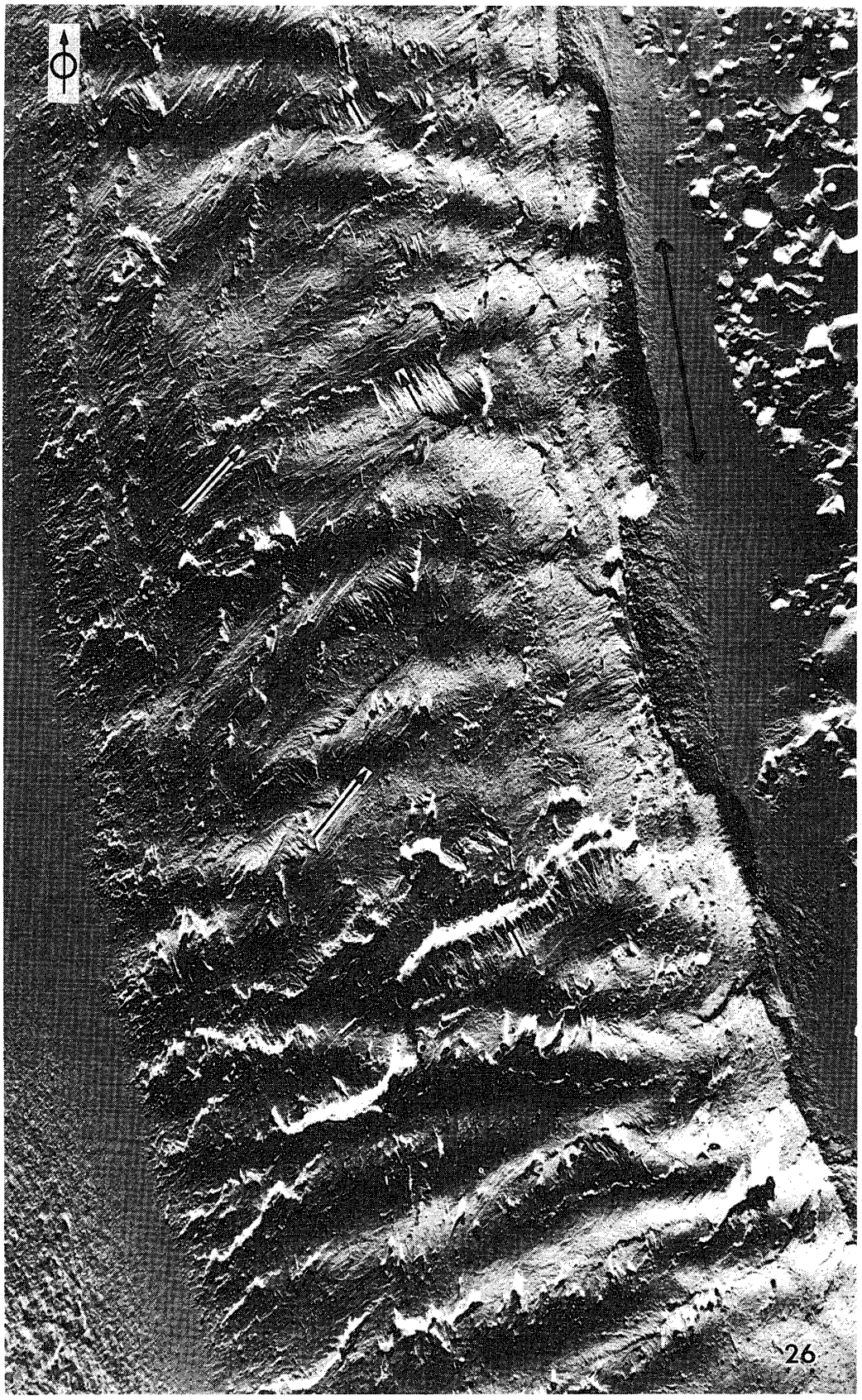
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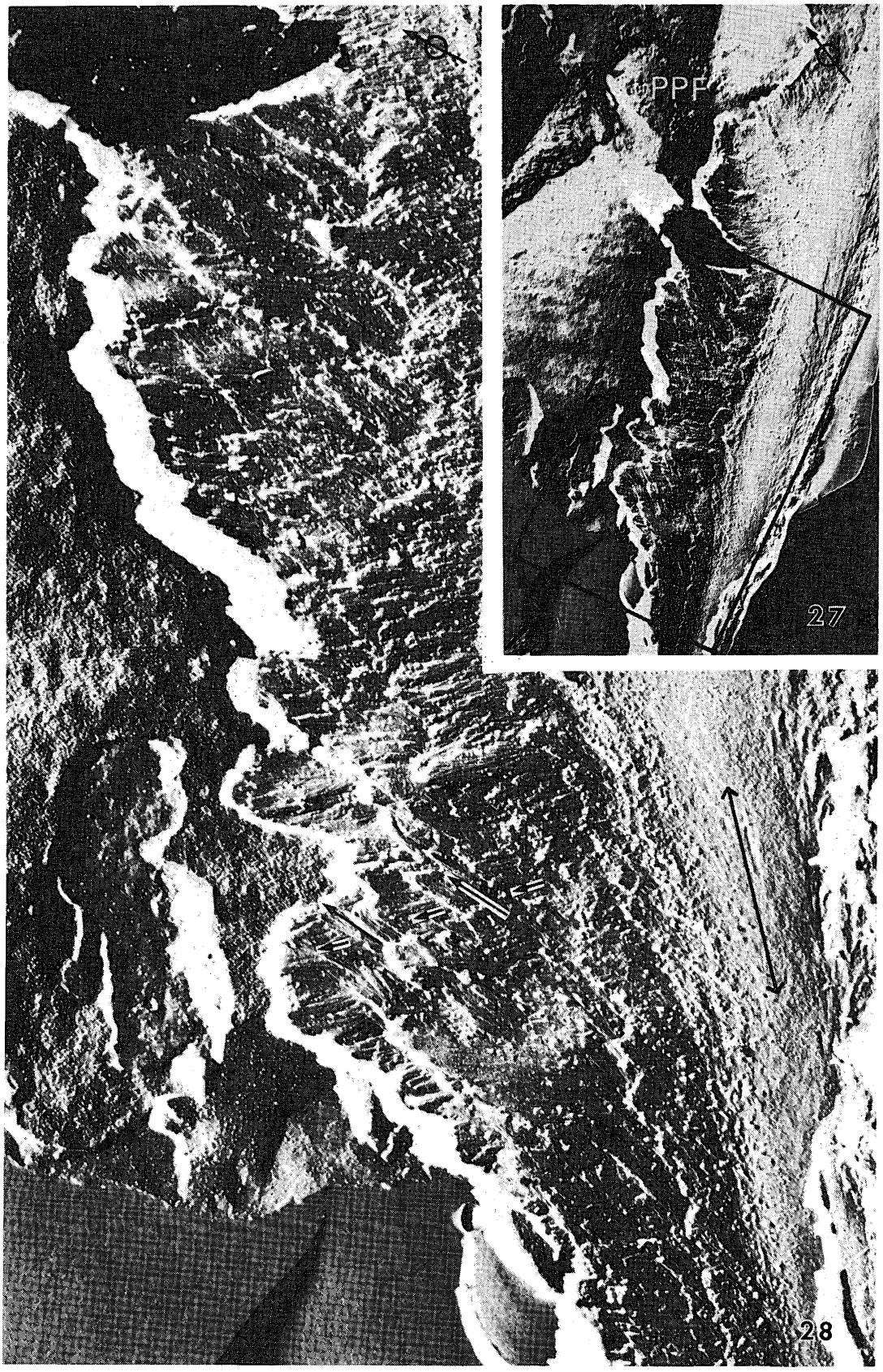
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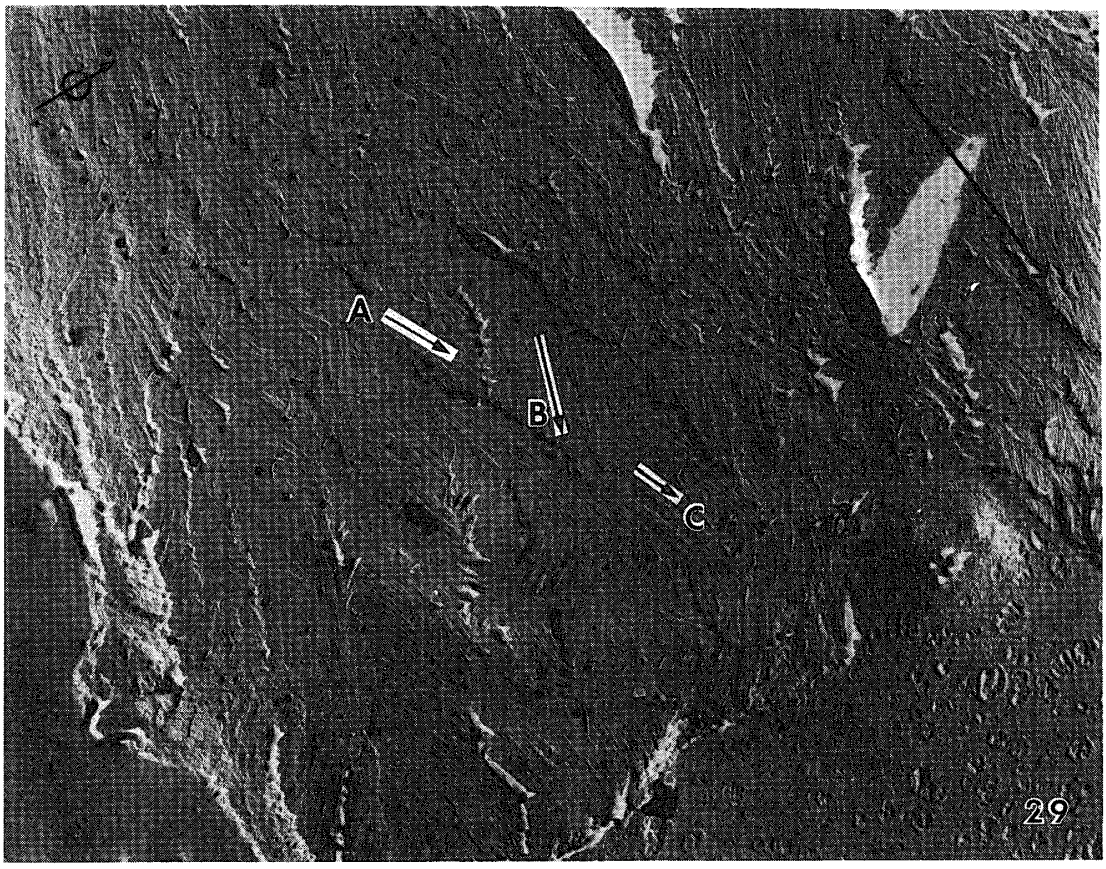
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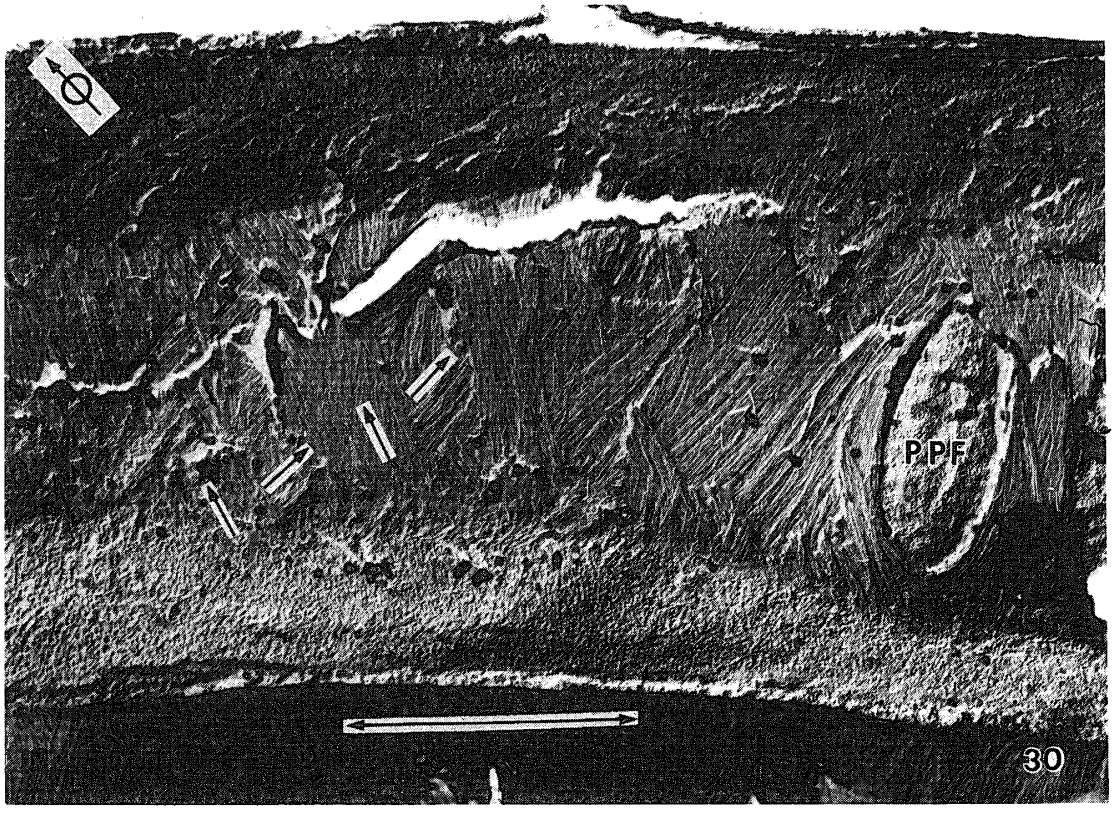
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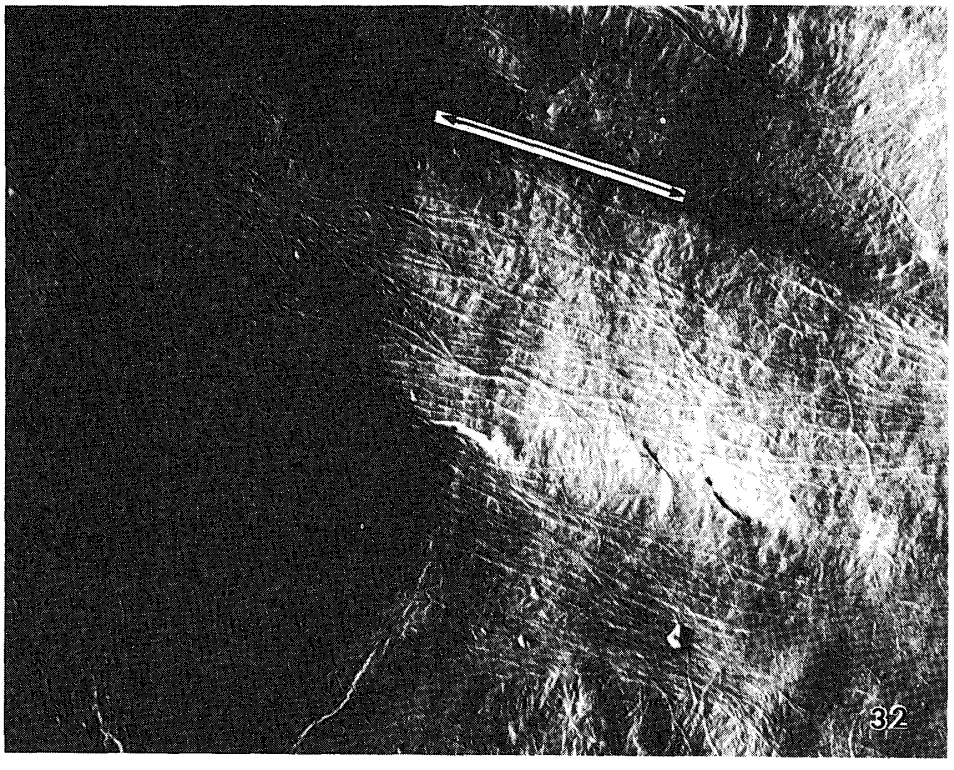

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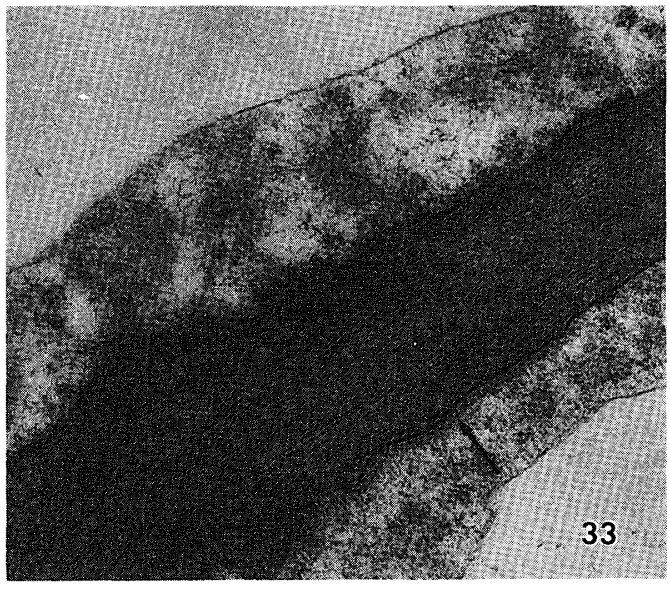
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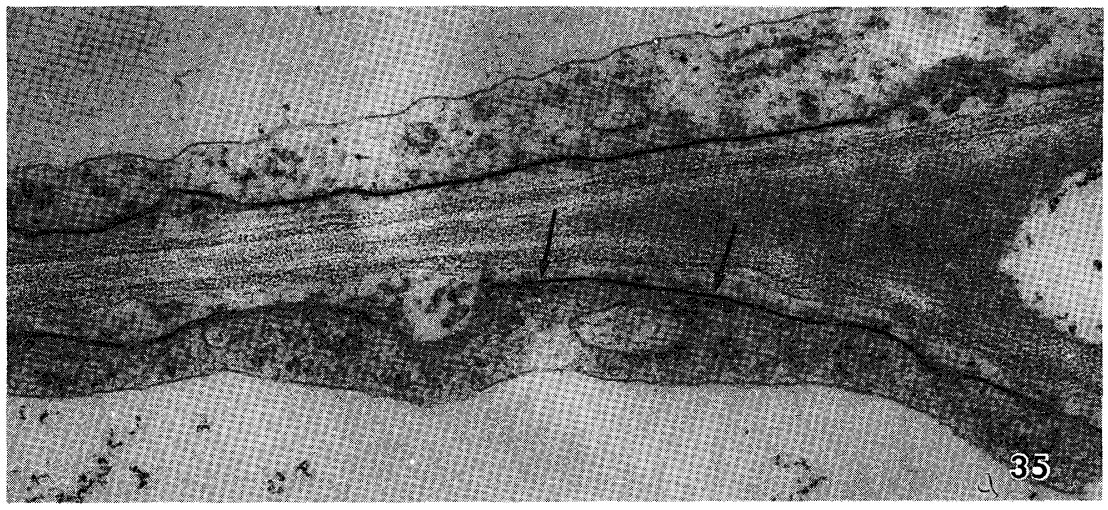
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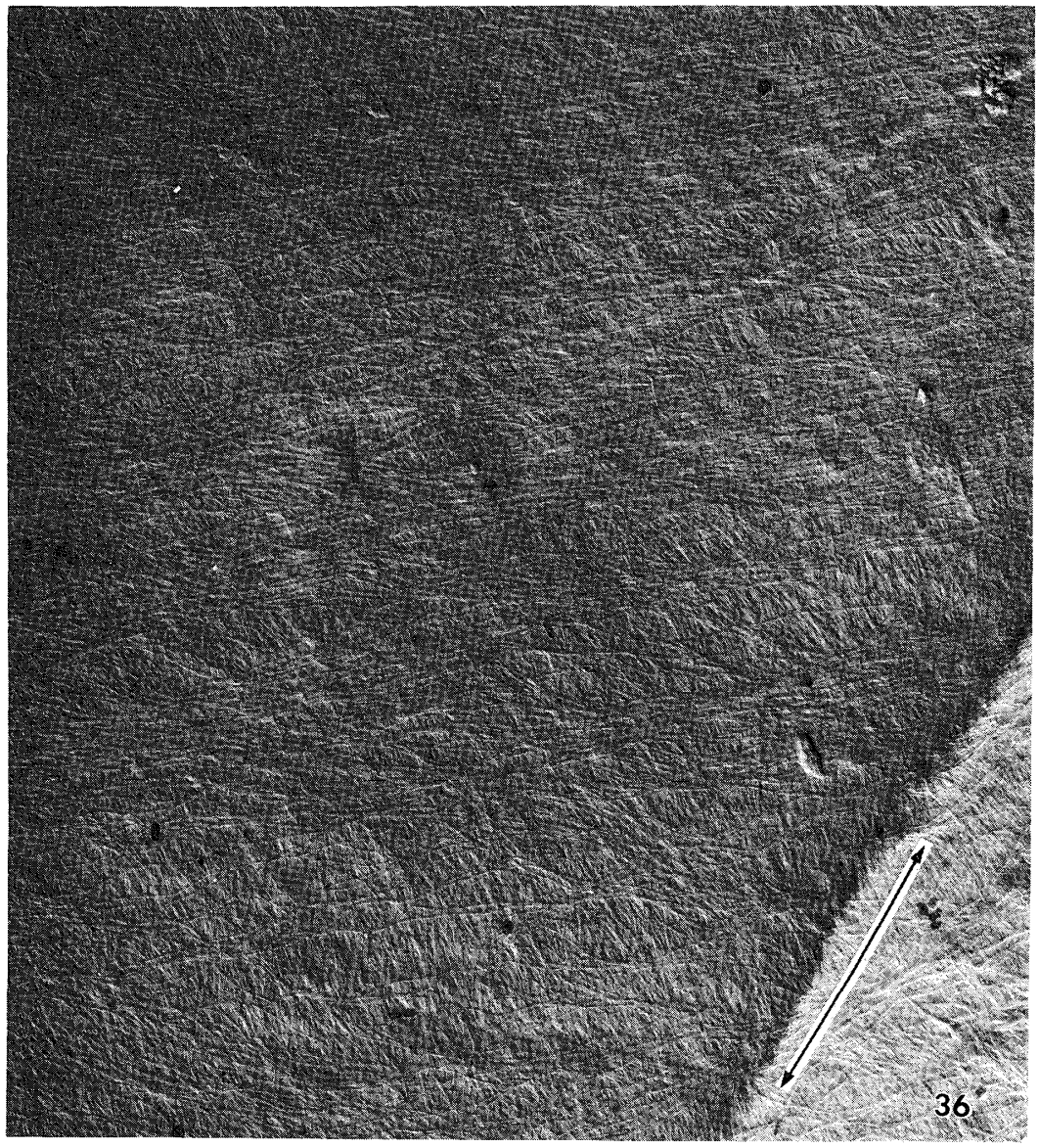
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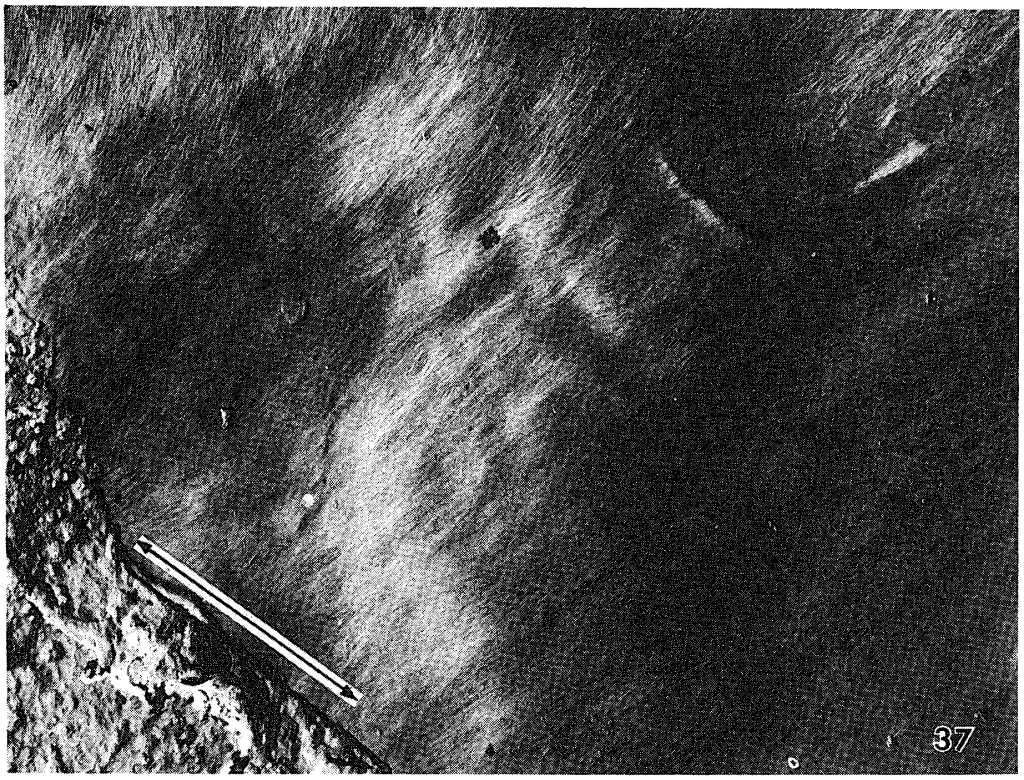
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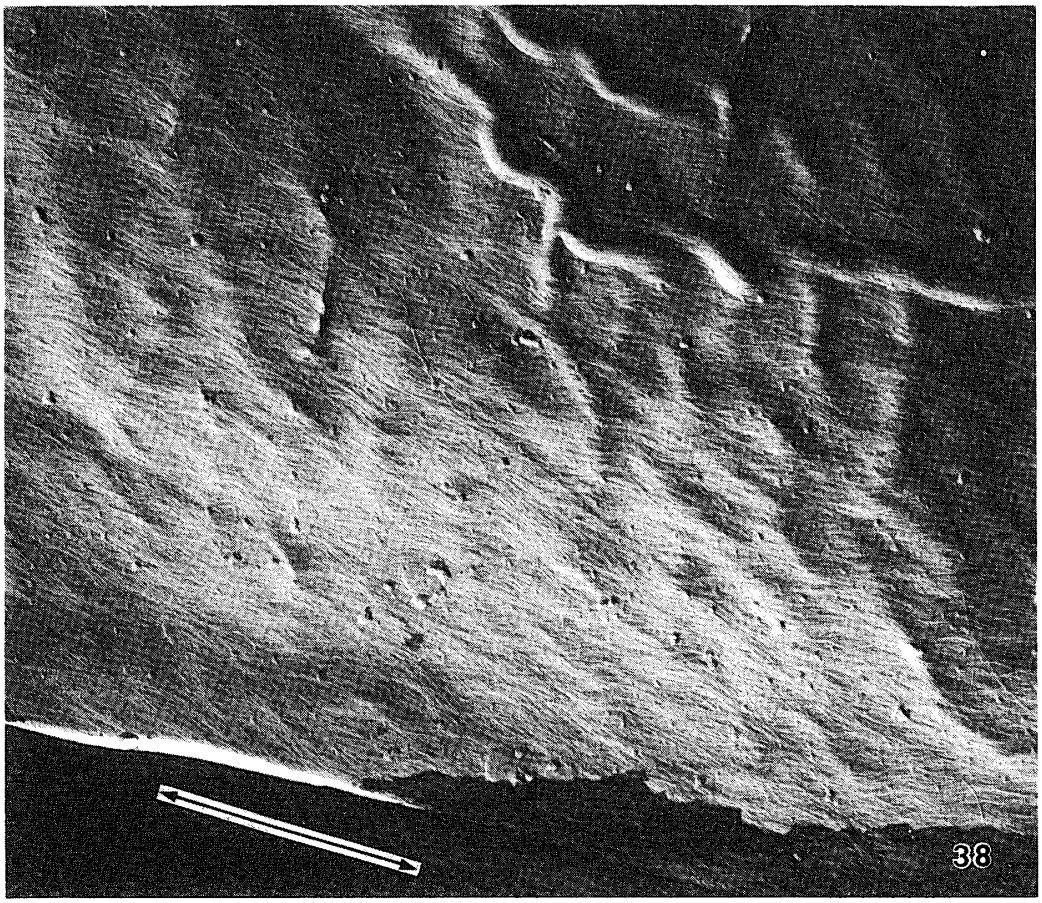
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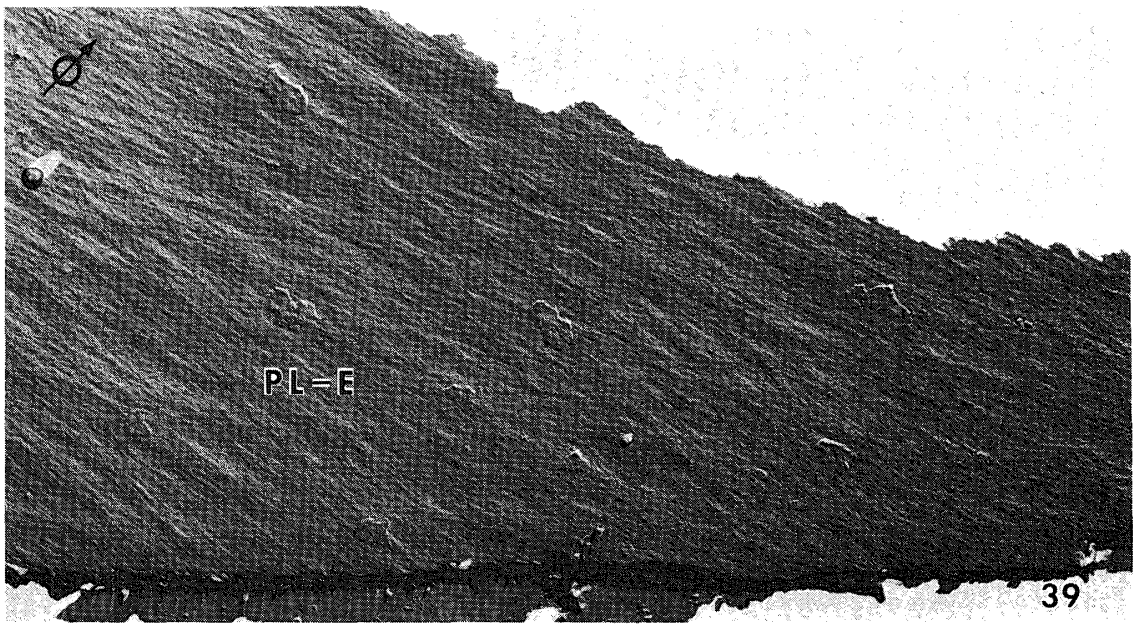
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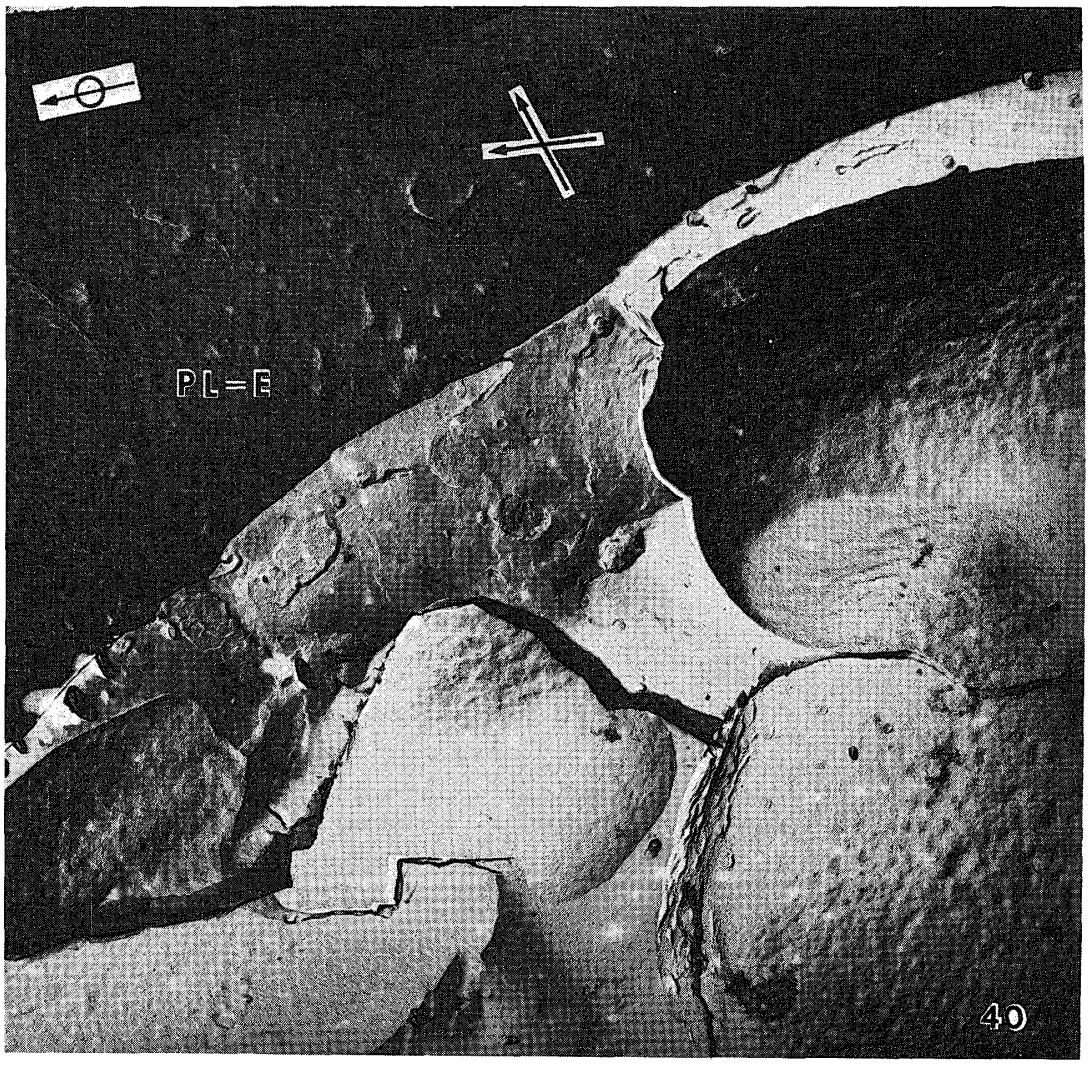
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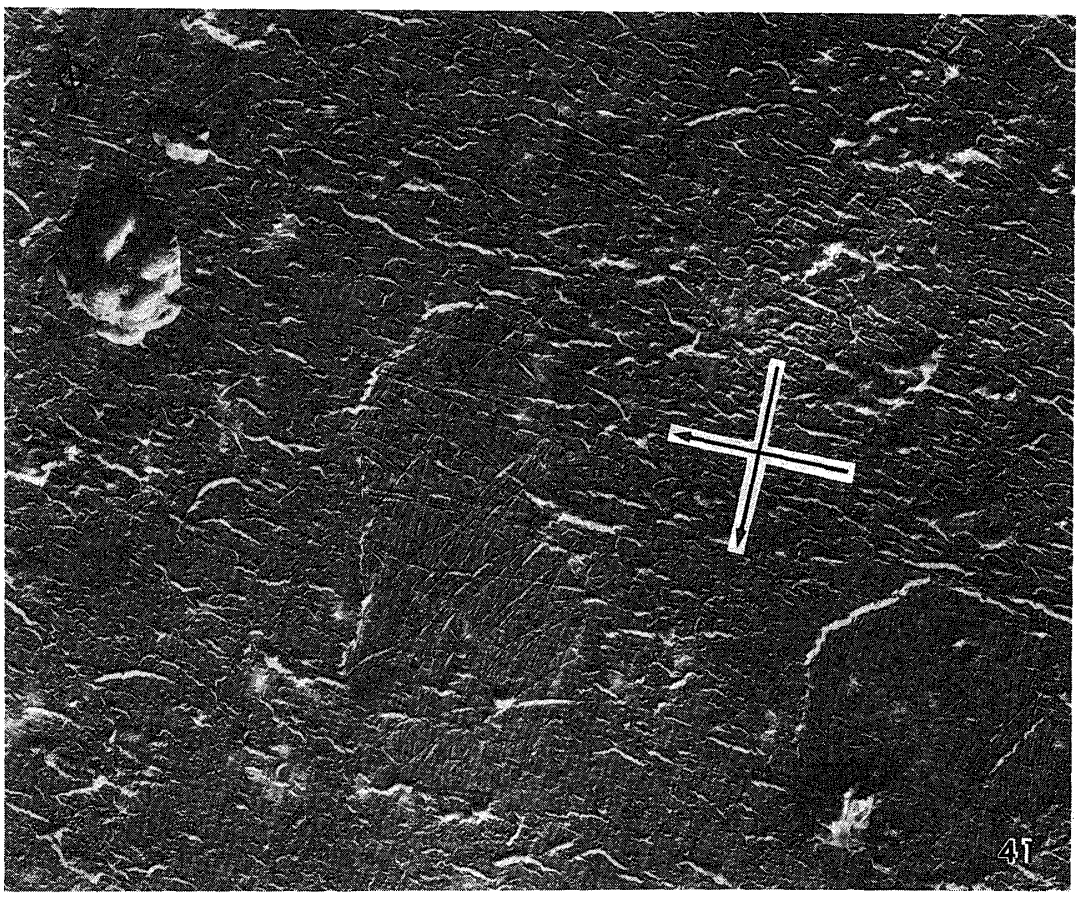

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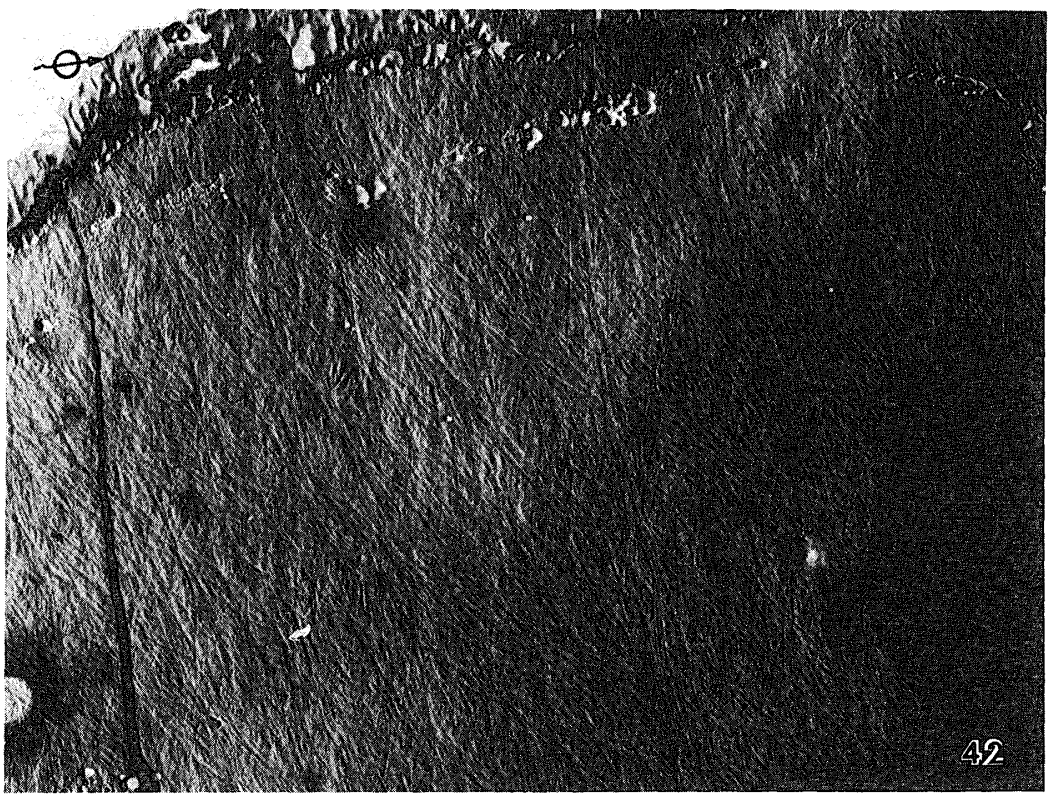

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