Histochemical observation of wood attacked by white rot fungi, *Coriolus versicolor* and *Cryptoderma yamanoi**

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白色腐朽材の組織化学的観察*

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CONTENTS

Introduction

Among many microorganisms capable to deteriolate wood, basidiomycete fungi are the most important because of their high capability of decaying wood cells. They are divided into two major groups, brown rot and white rot fungi, mainly in accordance with whether they can decompose lignin in wood and use it as a nutrition, or not.[9]

The white rot fungi, furthermore, can be divided into two types, white pocket rot and simultaneous rot fungi. The former type, e. g., *Trametes pini*, starts to metabolize lignin and hemicellulose; the cellulose fraction is degraded only at a later stage, leaving white fibrous remains. On the other hand, the latter, e. g.,

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Coriolus versicolor, is capable to decompose all components of a lignified cell wall at nearly the same rate[20),21),22)].

The morphological features of a wood cell wall attacked by fungi have been described by various authors for a long time, but there are only few reports about histochemical ones. Loss in weight of wood substance has been widely used as a measure of decay to combine both results of the chemical and anatomical changes. However, it seems to be somewhat unreasonable to use the weight loss to connect them, unless there is a respectable uniformity in the stage of decay within a specimen used.

In fact, it was often observed under the microscope by the present authors that cells intact as to their morphology were found closely in the neighbourhood of cells severely destroyed by fungous attack. Furthermore, it is well known that enzymes of white rot fungi are able to diffuse into a wood cell wall only for a limited distance and thus act within a minute area which can be distinguished with rather high resolution of the microscope[6),7),27),28),29)].

AUFSESS et al.[1),2),3),23)] applied fluorescence (FL) microscopy to histochemical study of decayed wood, and found that a clearly contoured change of colour occurred in the parts affected by degrading enzyme. They proposed that this offered an easy method to distinguish the white rot type from brown since a distinct colour change under the FL microscope was observed in the white rot but not in the brown. They considered that these changes of colour resulted from decrease of the lignin content of the cell wall, refering to the fact that the same change of colour occured in gelatinous layers of beech and oak tension woods for which researches were made by them. Recently FUKAZAWA et al.[10),11)] found that such a change of colour under a FL microscope appeared in both types of rots, white and brown, with some additional observations of the corresponding materials using ultraviolet (UV) microscopy. They described that this phenomenon might be due to the result of decomposition of polysaccharides, and/or a release of lignin.

This study aims to clarify the cause of the red discolouration, visible under the FL microscope, through further observations of the changes of cell wall components by fungous enzymes, using light and electron microscopy. At first, an observation with ordinary light microscopy was made with help of some dyes to detect the stainability of them at the red discolouration zone stated above. UV microscopical observation was made to compare the lignin content with the results obtained using the FL microscopy and several dyes described above, followed by UV microspectrophotometry, aimed at the investigation of the nature of lignin in the red zone. Wiesner colour test was also made to detect the relation of particular groups in lignin. In electron microscopy, specimens were prepared with potassium permanganate for lignin, and ruthenium red to detect the region affected by fungous enzyme concerned with polysaccharides.

Materials and methods

Two species of fungi as described below were selected for this study. Their

decaying patterns have been known to be different from each other, although the red discolouration in a particular area under the FL microscopy is expected to be common in both fungi. Heartwood of Ezomatsu (*Picea jezoensis*) decayed by *Coriolus versicolor* and that of Akaezomatsu (*Picea glehnii*) attacked by *Crypto-derma yamanoi* were used for this experiment. The former samples were provided from the Hokkaido Forest Products Research Institute, where they were incubated with *Coriolus versicolor*, on sand block culture of glucose peptone media at 26°C for several weeks after sterilization[9]. The latter samples were obtained from the trunk wood, opposite to the portion where a fruit body located, of a living Akaezo-matsu tree, grown in the Tomakomai Experiment Forest of Hokkaido University.

All of these samples were fixed and stored in FAA II (formalin : acetic acid : ethanol : water = 6.5 : 2.5 : 70 : 30). They were then cut into small cubes of 1 mm per each end, and dehydrated through a standard alcohol series and embedded in methacrylate for the optical microscope examinations. Sections of 1 μm in thickness were cut off the cubes on an ultramicrotom (LKB type 8802A), stained with acridine orange, a kind of fluorochrome, in 1% solution of citric-acid-phosphate buffer pH 6.0 for two hours, for observation by FL microscopy (Carl Zeiss, West Germany). Of various kinds of basic dyes, such as safranine, basic fuchsin, toluidine blue O, methylene blue, nile blue, and also of acidic dyes, such as fast green, acidic fuchsin, fluorescein sodium, each was used, staining the sections of 1 μm thickness in 1% aqueous solution for two hours, for ordinary light microscopy. Same thickness sections were also used for Wiesner colour reaction in standard staining method described by JENSEN[12].

Thin sections of 0.5 μm in thickness were used for the observation by ultraviolet microscopy (Carl Zeiss, West Germany Type MPM-01). UV microphotographs were taken on an ordinary commercial film at a wave length of 280 nm. UV microspectra were also taken in a wave length range of 250 nm to 320 nm.

Test specimens cut into small pieces for transmission electron microscopy were stained with 1% aqueous potassium permanganate for one hour or with 1% aqueous ruthenium red for 24 hours. After that they were dehydrated through a standard ethanol series after washing in running water overnight, and then em-bedded in Spurr's low viscosity epoxy resin. Ultra thin sections of 400–600 Å were cut and mounted on carbon coated grids. The transmission electron micro-scope used is the model JEM-100 C made by Japan Electron Optics Laboratory Co., Ltd.

Results and discussion

1) Fluorescence microscopy

Coriolus versicolor began to attack the tracheid cell wall from its inner suface and put the attack forward gradually to the middle lamella, showing in a thinning of the wall clearly visible under the microscope. The colour contrast in the FL micro-scopy between the attacking and sound zones was remarkable in a particular cell wall attacked (Photo 1), in agreement with the results reported by FUKAZAWA et al.[10]

In wood attacked by *Cryptoderma yamanoi*, the red discolouration zone appeared surrounding the dark green colour region (Photo 2) which corresponded to the completely delignified region observed with UV microscope, to be mentioned later (Photo 14). The attack of this fungus started to delignify the cell wall from the lumen side and progressed gradually toward the middle lamella. The thickness of the cell wall did not change in the light microscope observation, in contrast to the occurrence of thinning under the same microscope in the case of *Coriolus versicolor*.

Acridine orange (AO) is a sort of fluorochrome and metachromatic basic dyes. In regard to its staining nature, it has been said that AO fluoresces with red light when its cations exist as dimers and polymers, while as monomer it fluoresces with green[15)19)]. Since apparent red discolouration in the cell wall attacked by the fungi occurred in this experiment as stated above, it might be considered that AO molecules combined with substrates in the region affected by fungous enzymes.

Investigations of this sort of red discolouration in cell walls attacked by fungi have been reported, as stated above in this paper. FUKAZAWA et al.[10)] have described that this was possibly due to the result of decomposition of polysaccharides and/or release of lignin from LCC components. However, it seems to be somewhat unclear that the cause of red discolouration at the region was attributed only to the increase of accessibilty of cell walls. It also might be considered here that some newly created functional groups, such as carbonyl etc., of cell wall components would be related to this phenomenon, and then AO, a basic dye, possibly combined electrostatically with the cation of the substrates; therefore, the following experiment was done to clear this with the help of various basic and acidic dyes.

2) Ordinary light microscopy with help of basic dyes

In woods attacked by both fungi, *Coriolus versicolor* and *Cryptoderma yamanoi* used in this experiment, all the basic dyes showed a distinct staining zone of the cell wall, corresponding well to the red discolouration zone observed in FL microscopy, as shown in photos 3-12. The distinctly stained regions, which corresponded to the *degrading zone* of the cell wall by fungous enzyme, had fairly stable depth into the cell wall; in wood attacked by *Coriolus versicolor*, it was ca. 0.5 μm in depth along the inner surface of tracheid walls, stained with basic fuchsin (Photo 3), and, in the case of *Cryptoderma yamanoi*, it was ca. 0.2 μm in depth between the delignified remains and the sound part of the cell wall, stained with basic fuchsin (Photo 4).

In particular the safranine (Photo 5), being widely used as an indicator of lignification in histochemical study of differentiation, showed a distinct stain arround the bore hole (Photo 6), where increase of lignin content was considered improbable because of no change of UV absorption (Photo 19 b). The toluidine blue O, a kind of metachromatic basic dye, showed violet colour in the degrading zone, similar colour tone to that at the differentiating cell wall before lignification, while the sound part showed blue. Other basic dyes such as methylene blue and nile blue, showed the same stainability as basic fuchsin.

On the other hand, acidic dyes, such as fast green, acidic fuchsin, fluorescein sodium, did not show any distinct stain zone both under ordinary light and FL microscopy. It can be suggested that basophil functional groups such as acidic groups were created in the cell wall at the region distinctly stained with the basic dyes used.

3) **Ultraviolet microscopy** (280 nm wave length)

The experiment was made to investigate the relationship between the distinct staining of cell wall with the basic dyes used and lignin content there. In wood attacked by *Coriolus versicolor*, no significant difference found between the degrading zone near a completely degraded and disappeared part of cell wall or bore hole and the sound part, in UV (280 nm) absorption of the cell wall (Photo 13). Thus it was considered that the lignin content is not immediately related to the distinct stain with basic dyes, providing an agreement with the results reported by FUKAZAWA et al.[10),11)] In wood attacked by *Cryptoderma yamanoi*, complete delignification started from the lumen side and progressed gradually inward (Photo 14). Slight delignification occurred in the region corresponding to the red discolouration, in contrast to the case of *Coriolus versicolor*. Delignification within the S 2 layer in isolation from the inner surface of the cell wall in some distance was rarely found, showing a tapered end (Photo 15), similar to the cavity formed by soft rot fungi. As shown in Photo 16, a series of delignified spots was observed on radial walls of tracheids, adjacent to a ray from which delignification appeared to penetrate into tracheid walls.

4) **UV microspectrophotometry**

It was evident that the attack of *Cryptoderma yamanoi* progressed with gradual delignification of the tracheid wall from its lumen side toward the middle lamella, and a slightly delignified zone occurred, between degraded remains and sound part; this zone actually corresponded to the distinct staining zone by basic dyes used. This experiment was made to investigate the change of nature in the lignin fraction of the cell wall in this degrading zone.

In comparision with the sound part, the UV spectral curve of this degrading zone markedly showed increasing absorption above 300 nm wave length, while decreasing at 280 nm (Fig. 1). Absorbance curves were obtained at wave lengths from 280 nm to 320 nm at the interval of 10 nm, by means of stage scanning, in order to investigate the local changes of UV absorption across the cell wall. They showed clearly that the peaks in the absorbance curves in the degrading zone appeared above 290 nm, and disappeared at 280 nm (Fig. 2, vertical line).

Refering to the data obtained with chemical methods about degraded lignin, reported by KIRK et al.[13),14)], it can be noticed here that increase of UV absorption above 300 nm wave length shows creation of functional groups such as α-carbonyl groups in the lignin fraction by fungous enzyme.

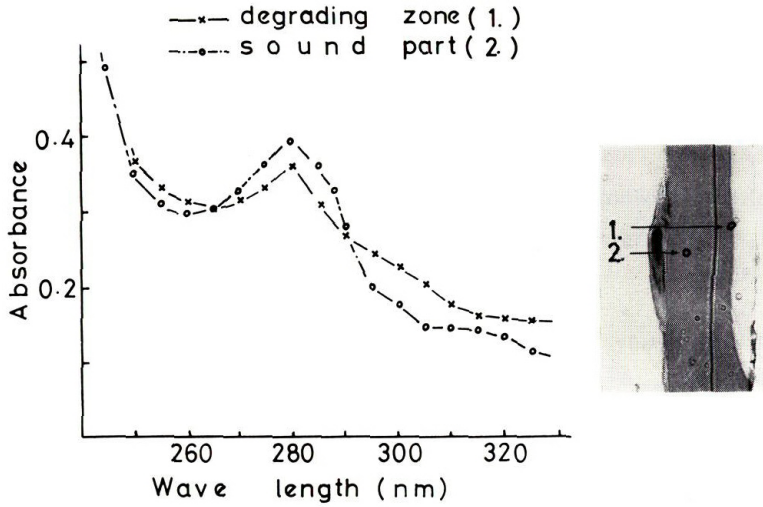

Fig. 1. UV spectral curves for the S2 of Akaezomatsu tracheid attacked by *Cryptoderma yamanoi*, in sound part and degrading zone.

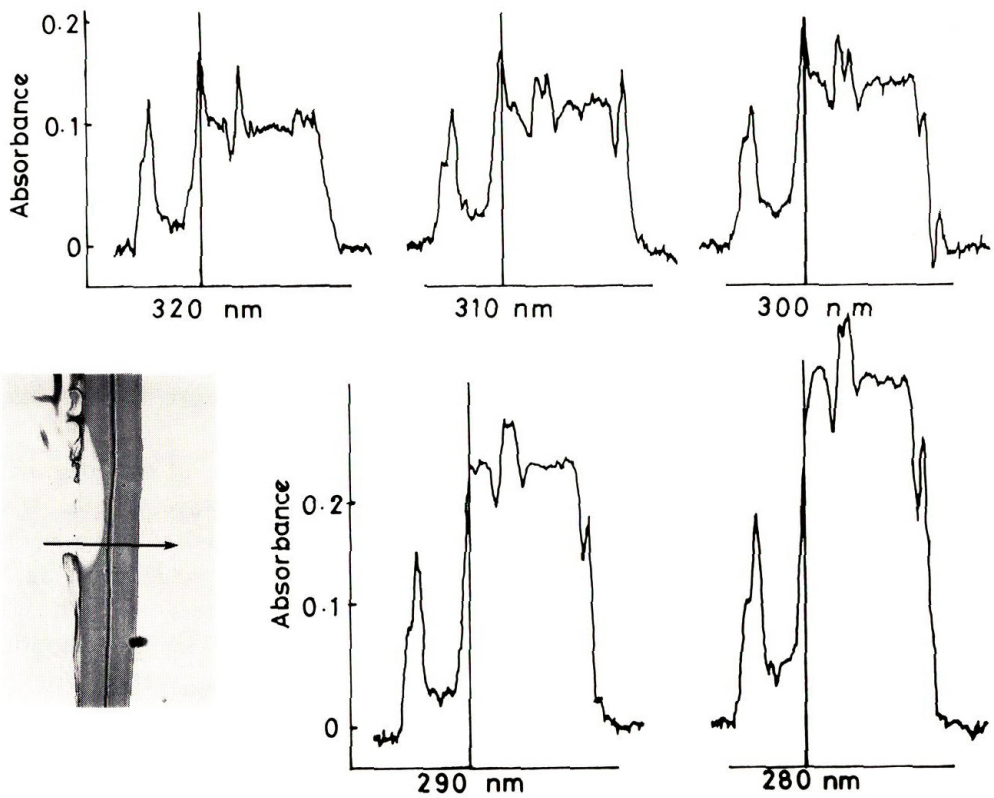

Fig. 2. Absorbance curves at each wave length, from 280 nm to 320 nm, using stage scanning method. The arrow in UV photograph (280 nm wave length) indicates the direction of scanning spot.

5) Colour reaction of Wiesner reagent

It is well known that colour reaction of phloroglucinol-hydrochloric acid depends on the presence of coniferyl aldehyde groups in lignin[24]. This experiment was made to investigate the relationship between the change of UV spectra and the presence of such groups in lignin of the degrading zone. In wood attacked by *Coriolus versicolor*, it was found that no difference between degrading zone and sound part in this colour reaction occurred even in the area where thinning appeared remarkably (Photo 17).

In the case of *Cryptoderma yamanoi*, this colour reaction was slightly weak at boundary (degrading zone) between delignified remain and sound part, judging from the obscure outline (Photo 18), and it was also found weaker arround the bore hole in some extent than that in the sound part (Photo 19 a).

It can be suggested from this experiment that the attack of *Cryptoderma yamanoi* degraded lignin fraction to a certain extent in advance of the delignification of the cell wall, because there were no obvious changes in UV absorption arround bore holes at 280 nm wave length (Photo 19 b).

6) Electron microscopy

It was evident in the light microscopical observation that the degrading zone by fungous enzyme is restricted to a certain extent into the cell wall, ca. 0.5 μm depth in *Coriolus versicolor* and ca. 0.2 μm in *Cryptoderma yamanoi* (Photos 3 and 4). Taking into account of the results described above, an electron microscopical experiment was made to obtain more information about cell walls attacked by *Cryptoderma yamanoi*.

The technique employed in this experiment was actually corresponded to the common histochemical techinque in light microscopy, staining with two electron dense solutions and obtaining the information about change of cell wall components from their stainability.

a) Observation of stained specimen with potassium permanganate

This reaction, which is in part the basis of the Mäule test, has been studied by various authors, and it was known that potassium permanganate is reduced to manganese oxides in the presence of lignin[5]. The authors, therefore, presumed that a staining effect was obtainable due to the electron opacity of manganese which is a complex with lignin. It was shown through this experiment that there were no changes of stainability arround the bore holes, showing an agreement with the results obtained with UV microscopy described above (Photo 20). It was evident from this result that weakness of the Wiesner colour reaction arround the bore hole was due to the degraded lignin, not to decrease of lignin content. Photo 21 illustrates the margin of a bore hole, showing a fine brush-like feature of the cell wall components destructed, combined with manganese particles.

b) Observation of stained specimen with ruthenium red

Ruthenium red has been used as a stain for pectin for a long time. However, it is now known that not only pectin, but also all polysaccharides containing

carboxyl groups are stained by this reagent[26]. The crystal-structure of this reagent and stereochemistry of its pectic stain were reported by STERLING; he summarized as follows, the staining group is composed of the ruthenium ion and the associated square planar complex of four ammonia molecules. The staining site in the host molecule must have two negative charges 4.2 Å apart and space to accommodate the staining group, lying with its plane perpendicular to the axis between these charges[25].

In this experiment, ruthenium red was used as an electron staining medium to detect such staining sites created by oxidative and/or hydrolysis degradation by fungous enzymes. Photo 22 was taken in the position corresponding to the region completely delignified, evidenced with UV microscopy. Microfibrils like in lamella shape are exposed where the lignin was removed. Ruthenium red seems to stain these microfibrils involving degraded cellulose (average degree of polymerization 180)[16),17),18)] by fungous enzymes. As shown in Photo 23, cell wall components are visible in parallel with the orientation of microfibrils in the S 2 layer as the result of staining with ruthenium red. Gradual increase in depth (in longitudinal direction) of their penetration from the middle lamella inward is also clearly visible in this photograph. It can be presumed from this result that enzyme action at the lumen side differs from that of the bore hole. However, further investigation of this aspect is necessary.

Conclusion

It is an important problem for the histochemical study of decayed wood, to detect the area being affected by fungous enzyme and to clarify the change of cell wall components occurring there.

The FL microscopy used for this study was able to detect the degrading zone of the cell wall through a discolouration into red which should be considered as an indicator of the change occurring there. This red discolouration zone was found along the inner surface of the tracheid wall attacked by *Coriolus versicolor* in a depth of ca. 0.5 μm, as shown in photo 3. In the case of *Cryptoderma yamanoi* the zone appeared in between the delignified region and the sound part of the cell wall in depth of ca. 0.2 μm, as shown in Photo 4.

Experiments carried out to clarify the change occurring in/about the red discolouration zone were described and briefly discussed above, and gave certain progress for the study of this item. Increasing stainabilty of basic dyes for the area corresponding to the red discolouration zone under the FL microscopy was obtained apparently.

The cause of the red discolouration in cell walls attacked by both fungi was not found to be same for both. In the wood decayed by *Coriolus versicolor*, the change of a particular cell wall component was not the major cause of it, but the simultaneous affection of cell wall substances by the enzyme increased accessibility of the cell wall components for dyes used. On the other hand, in the case of *Cryptoderma yamanoi*, predelignification in the area resulted in the red dis-

colouration under the FL microscope.

Around the bore holes, red discolouration also appeared in wood decayed by both fungi used, particularly in attack by *Cryptoderma yamanoi*. It can be possibly presumed that the enzyme action of this fungus at the inner surface of the cell wall differs from the case of the bore hole. Further investigations are required for this point.

The authors are very much interested in what sort of phenomenon in ultrastructural level is progressing within the degrading zone defined with FL and ordinary light microscopy. Histochemical study using electron microscopy should be required to continue.

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摘　　要

カワラタケを接種し培地上で実験室的に腐朽させたエゾマツ心材と，エゾサルノコシカケにより生立木腐朽されたアカエゾマツ心材を，蛍光顕微鏡・紫外線顕微鏡及び透過電子顕微鏡を用いて組織化学的に観察した。方法及び結果を要約すると次のとおりである。

1) メタクリレート包埋した1μ切片をアクリジンオレンジで染色して蛍光顕微鏡で観察した。また同様な切片を塩基性色素及び酸性色素で染色して光学顕微鏡で観察した。Wiesnerの呈色反応もこの薄切片法を用いて分解能を高めることに努めた。紫外線顕微鏡用には0.5μ切片を作り，280 nmで写真撮影を行ない，また同顕微鏡光度計で紫外線吸収スペクトルを測

定してリグニンの量及び性質についての知見を得た。

2) エゾサルノコシカケ腐朽材をエポン包埋して超薄切片を作り，透過電子顕微鏡で観察した。試料を包埋の前に，リグニン染色剤として過マンガン酸カリウムを，また酵素の作用域を知るためのものとしてルテニウムレッドを用いてそれぞれ電子染色した。

3) カワラタケ腐朽材では，アクリジンオレンジ染色により内腔に沿い薄化した細胞壁部分やせん孔の周囲の部分に赤化が認められた。塩基性色素でも同じ部位が強く染色されたが酸性色素ではそれがみられなかった。紫外線顕微鏡での観察及び Wiesner 試薬の反応性からみて壁の分解に先立ちリグニンのみが選択的に強い酵素作用を受けることはないと判断された。

4) エゾサルノコシカケ腐朽ではカワラタケのような顕著な薄化は起こさずに細胞壁からスポット状にリグニンが逐次分解除去される腐朽経過を示す。アクリジンオレンジ及び塩基性色素はその部分を縁取るように細胞壁を強く染色する。紫外線スペクトル及び Wiesner 試薬の反応性からみて狭い範囲ではあるが前脱リグニン的作用が起こっていることが判断された。

5) アクリジンオレンジ及び塩基性色素の染色域の深さは両腐朽材とほぼ一定であり，カワラタケ腐朽材では約 0.5 μ，エゾサルノコシカケ腐朽材では約 0.2 μ である。これは菌の酵素作用域を示していると考えられ，この部分の微細構造レベルでの組織化学的研究が極めて必要である。

6) エゾサルノコシカケ腐朽材についての観察によれば，過マンガン酸カリウム染色では，せん孔周囲においてその染色性の低下が認められず，紫外線顕微鏡 (280 nm) によるリグニン量に変化なしとする知見と一致する。またせん孔の内表面は凹凸が激しい。ルテニウムレッドは菌の作用を受けた多糖類を染色することが明らかと成った。

Explanation of photographs

Note: The tracheid axis is vertical in all photographs (↕) except two, photos 22 and 23. All photographs show radial views.

Photo 1. A tracheid wall decayed by *Coriolus versicolor*, stained with acridine orange, showing the red discolouration along the inner surface of tracheid and completely degraded, disappeared (thinning) region (Ezomatsu, *Picea jezoensis*).

Photo 2. A tracheid wall decayed by *Cryptoderma yamanoi*, stained with acridine orange, showing the red discolouration occurred surrounding dark green region (Akaezomatsu, *Picea glehnii*).

Photo 3. A tracheid wall attacked by *Coriolus versicolor*, showing the completely degraded, disappeared (thinning) region (arrow 1), distinct staining zone which means degrading zone (arrow 2) and sound part of cell wall (arrow 3). Stained with basic fuchsin (Ezomatsu).

Photo 4. A tracheid wall attacked by *Cryptoderma yamanoi*, showing delignified remain (delignified region, arrow 1), distinct staining zone which means degrading zone (arrow 2) and sound part of cell wall (arrow 3). Stained with basic fuchsin (Akaezomatsu).

Photo 5. Tracheid walls decayed by *Coriolus versicolor*, showing distinct staining along the inner surface of cell walls and arround a bore hole. Stained with safranine (Ezomatsu).

Photo 6. A tracheid wall decayed by *Cryptoderma yamanoi*, showing three bore holes each of which is surrounded by dark circles due to stain with safranin. Stained with safranine (Akaezomatsu).

Photo 7. Tracheid walls decayed by *Coriolus versicolor*, showing the distinct staining zone along the inner surface of cell wall. Stained with methylene blue (Ezomatsu).

Photo 8. A tracheid wall decayed by *Cryptoderma yamanoi*, showing the hypha penetrating through the cell wall and distinct staining zone arround the bore hole. Stained with methylene blue (Akaezomatsu).

Photo 9. Tracheid walls decayed by *Coriolus versicolor*, showing the distinct staining zone with toluidine blue O, this dye is metachromatic one with which degrading zone is violet and sound part is blue in colour tone (Ezomatsu).

Photo 10. A tracheid wall decayed by *Cryptoderma yamanoi*, showing distinct staining zone in S2 layer, unstained in delignified remain. Stained with toluidine blue O (Akaezomatsu).

Photo 11. Tracheid wall decayed by *Coriolus versicolor*, showing distinct staining zone having uniform depth. Stained with nile blue (Ezomatsu).

Photo 12. A tracheid wall decayed by *Cryptoderma yamanoi*, showing the distinct staining zone corresponding to the front of progressive change of cell wall components. Stained with nile blue (Akaezomatsu).

Photo 13. Tracheid walls decayed by *Coriolus versicolor*, observed with UV microscopy (280 nm wave length). There is no significant difference of UV absorption between sound part and degrading zone (Ezomatsu).

Photo 14. A tracheid wall decayed by *Cryptoderma yamanoi*, observed with UV microscopy (280 nm wave length), showing the delignification progressing from the inner surface of cell wall (Akaezomatsu).

Photo 15. A tracheid wall showing the spot-like lacking of UV absorption in S2 layer in some distant from the inner surface of the cell wall, decayed by *Cryptoderma yamanoi*. Observed with UV microscope (280 nm wave length) (Akaezomatsu).

Photo 16. The ray crossings of tracheids showing small areas lacking UV absorption, decayed by *Cryptoderma yamanoi*. Observed with UV microscope (280 nm wave length) (Akaezomatsu).

Photo 17. Wiesner colour reaction of Ezomatsu tracheid walls, showing no difference of this reaction among near the bore hole, degrading zone and sound part of cell wall decayed by *Coriolus versicolor*.

Photo 18. Wiesner colour reaction of Akaezomatsu tracheid wall decayed by *Cryptoderma yamanoi*, showing delignified region (delignified remain) in S2 layer. The boundary between delignified remain and sound part was obscure.

Photo 19. Wiesner colour reaction of Akaezomatsu tracheid decayed by *Cryptoderma yamanoi*, being weak arround the bore hole (Photo 19 a), in comparison with UV photograph at 280 nm wave lenght in the same position (photo 19 b).

Photo 20. Tracheid wall decayed by *Cryptoderma yamanoi*, showing bore hole. Stained with potassium permanganate (Akaezomatsu).

Photo 21. Tracheid wall decayed by *Cryptoderma yamanoi*, showing a fine brush-like feature in the inner surface of a bore hole. Stained with potassium permanganate (Akaezomatsu).

Photo 22. Tracheid wall decayed by *Cryptoderma yamanoi*, showing the delignified region which is corresponding to that in Photo 14. Stained with ruthenium red (Akaezomatsu).

Photo 23. Tracheid wall decayed by *Cryptoderma yamanoi*, showing a bore hole, stained with ruthenium red (Akaezomatsu).

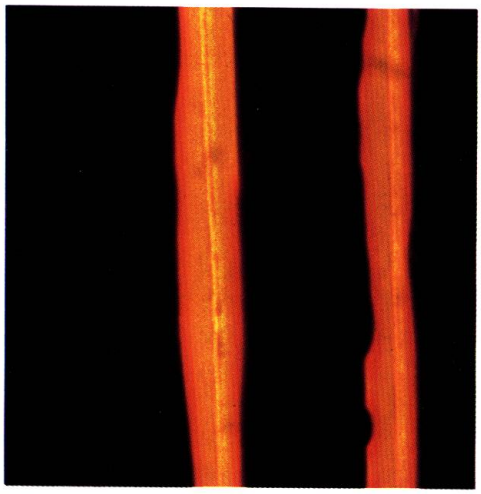
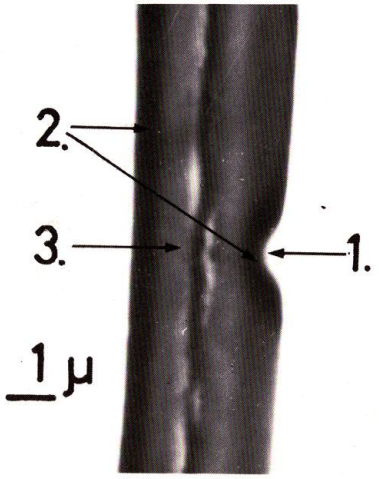


Photo 1

Photo 2



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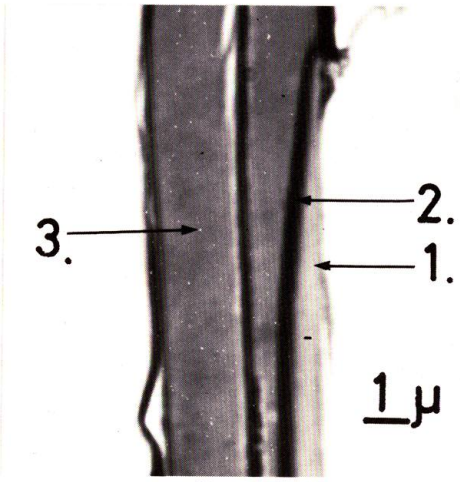

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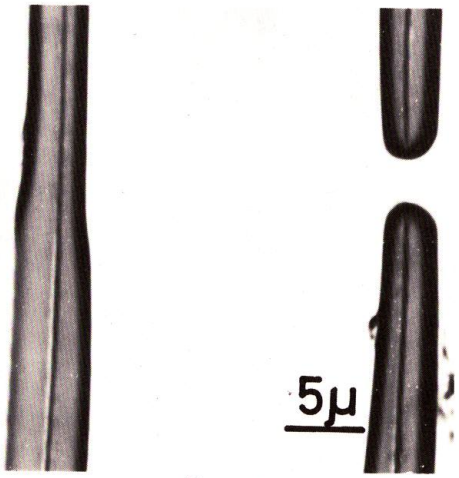

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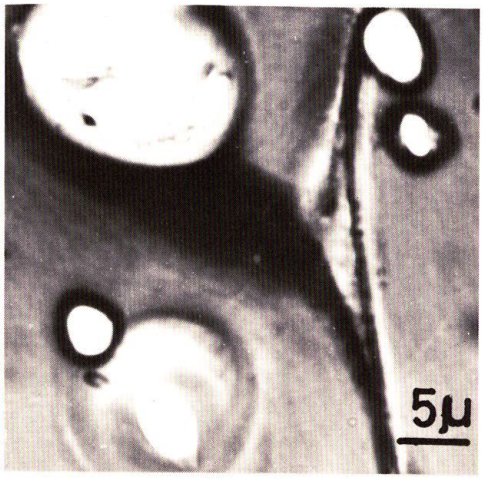

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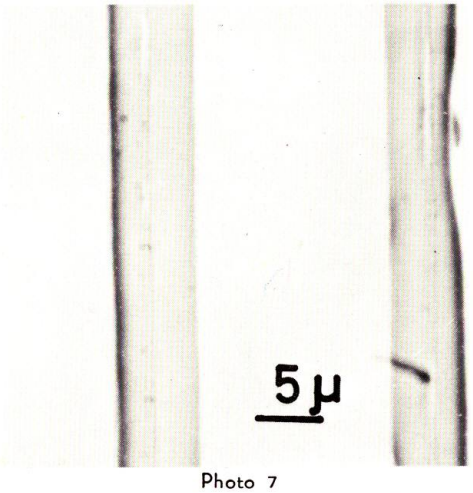

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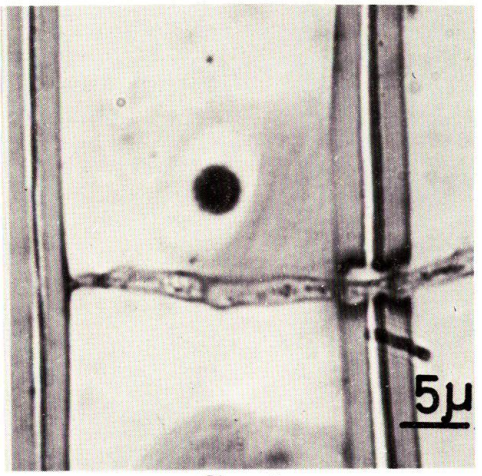

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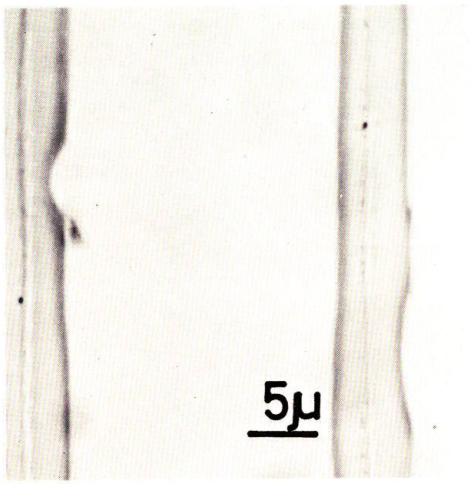

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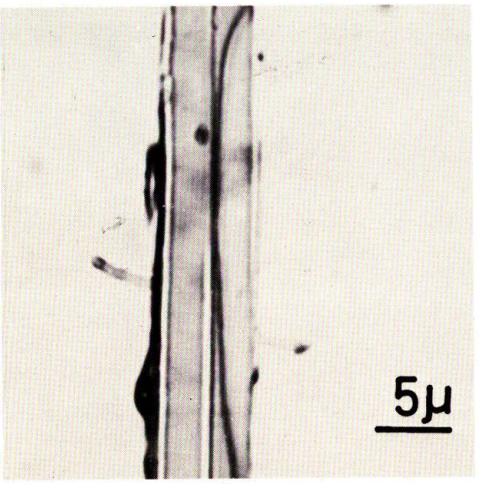

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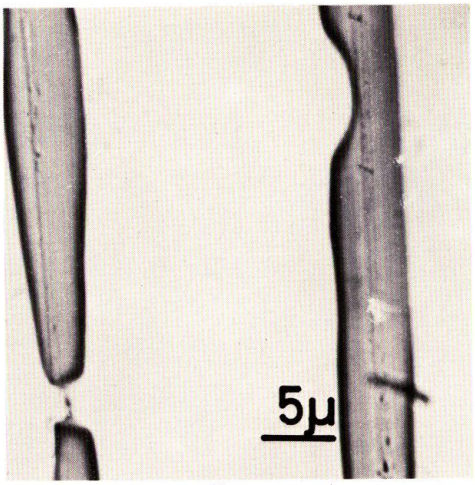

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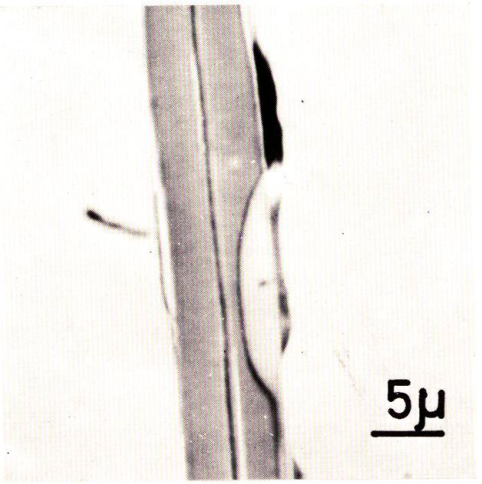

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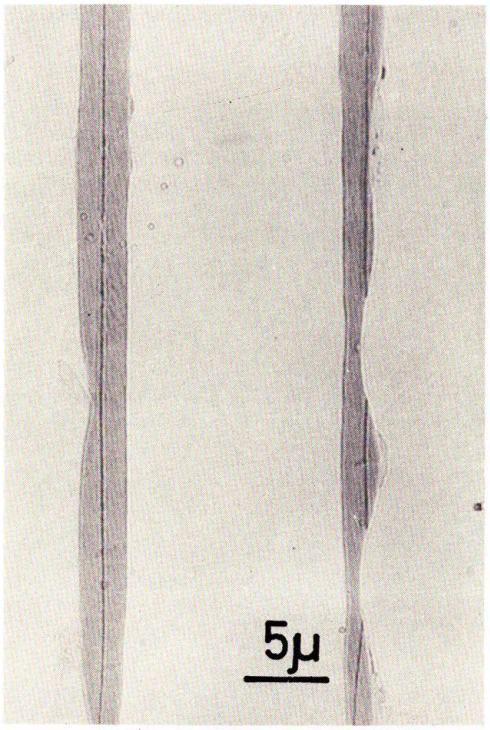

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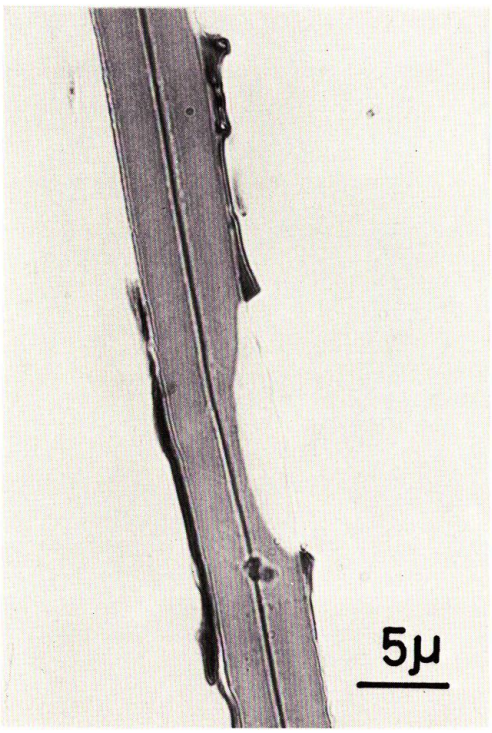
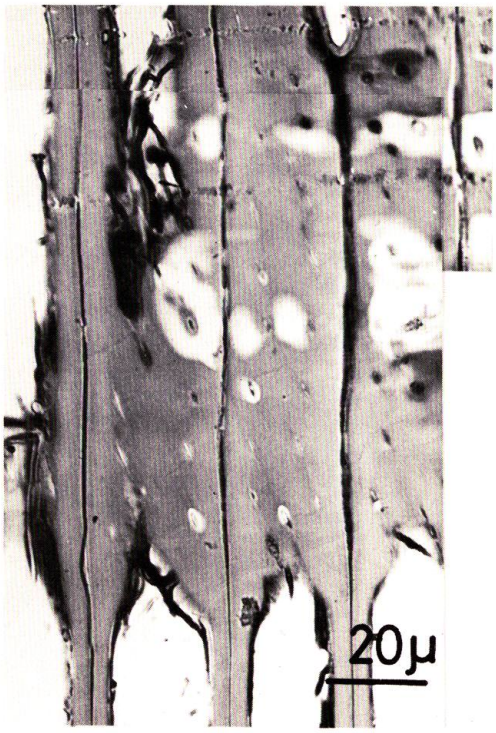


Photo 14

Photo 15



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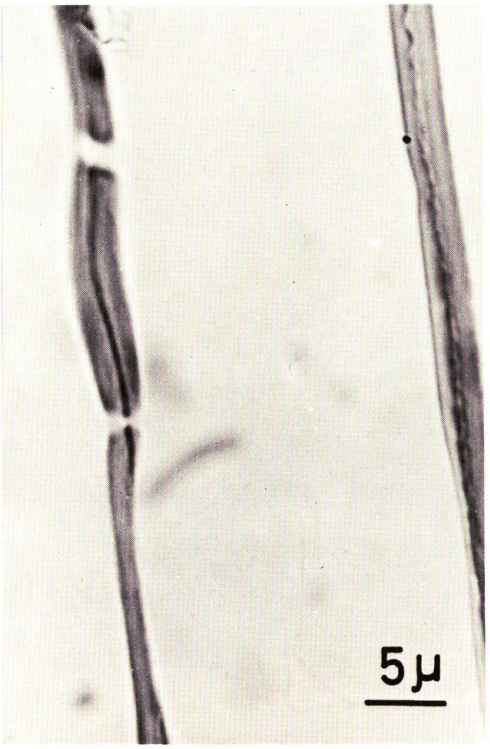

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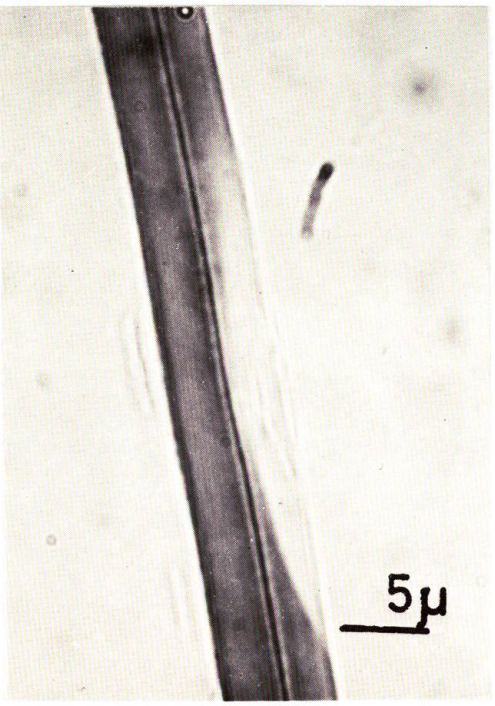

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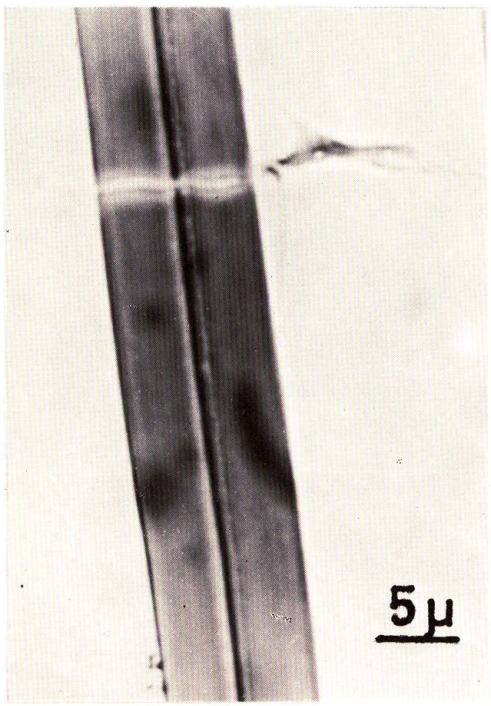
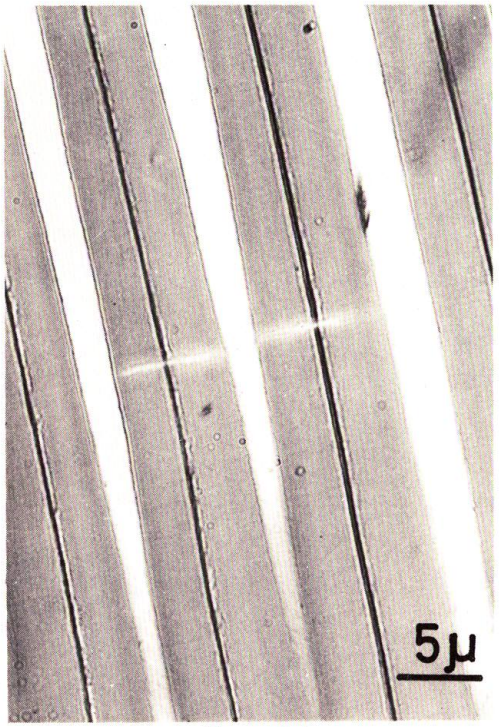


Photo 19-a



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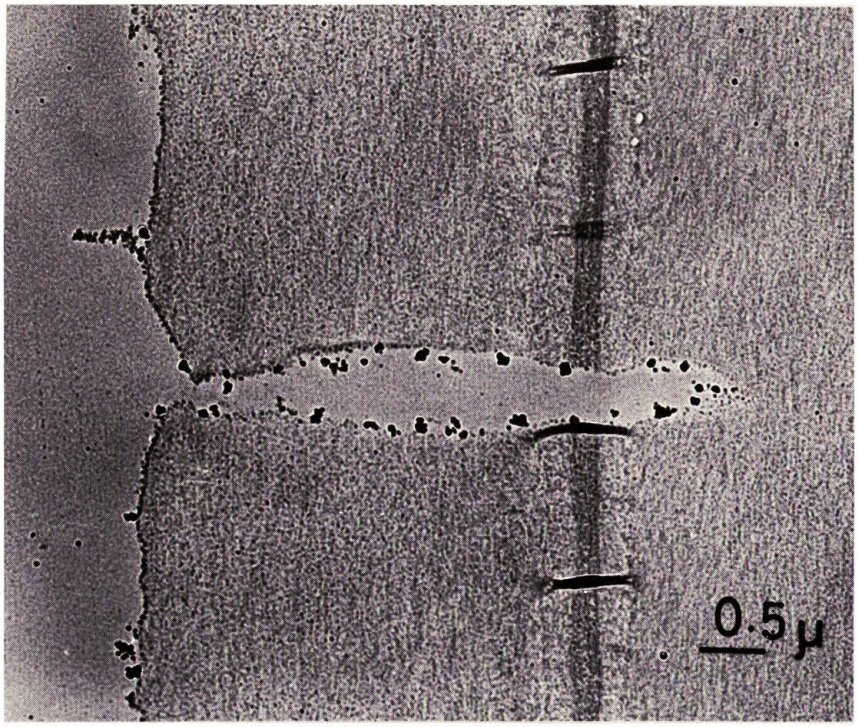

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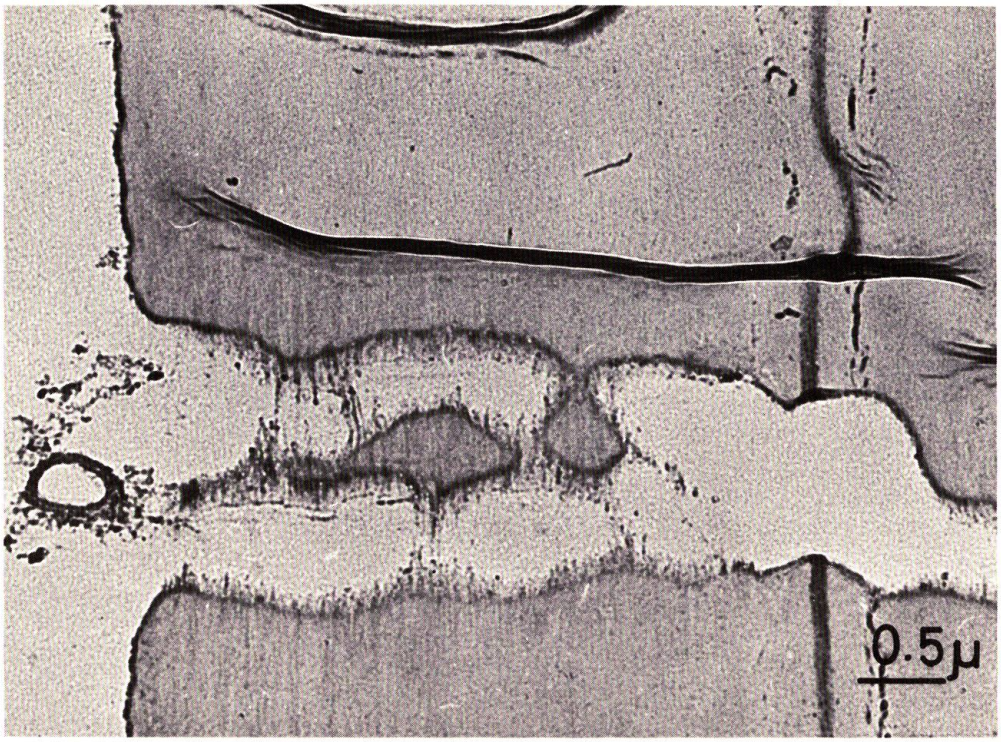

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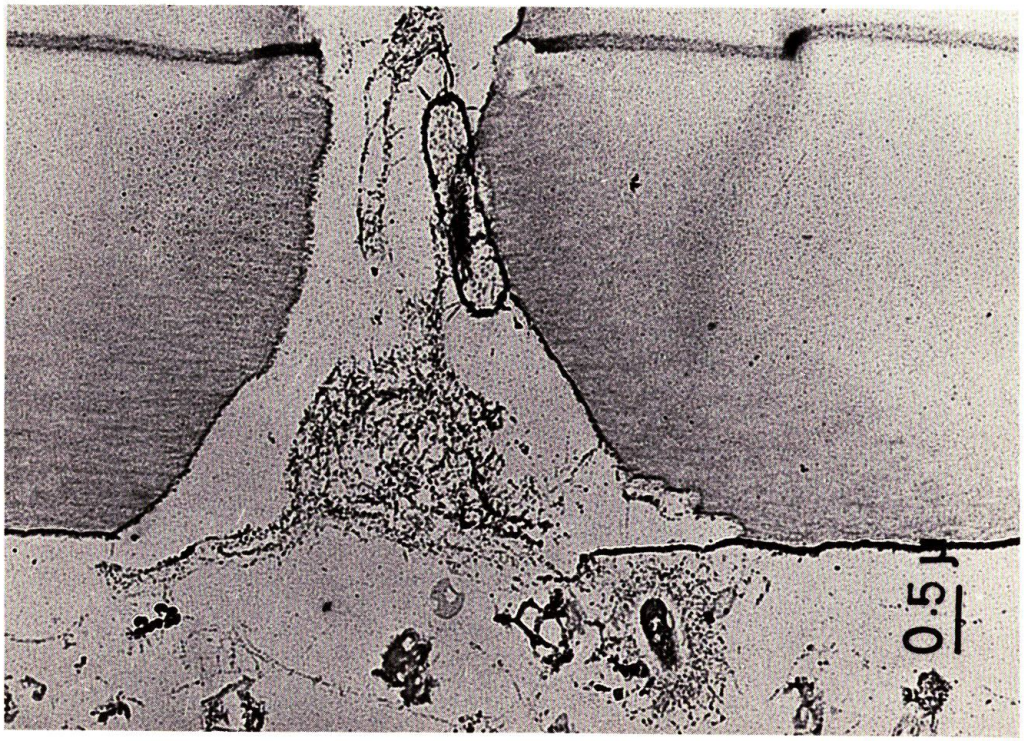

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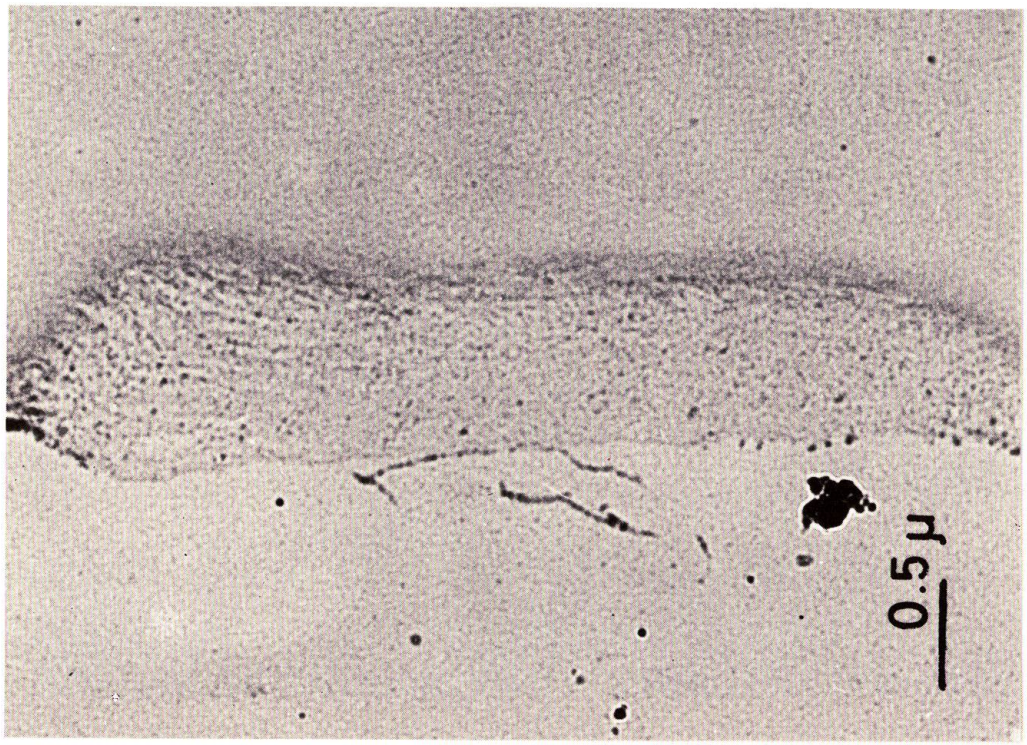

Photo 22