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Penetration of the Walls of Wood Cells by the Hyphae of Wood-Destroying Fungi

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BY

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INTRODUCTION

DECAY of wood results from the destructive action of certain fungi which convert its various components into substances suited to fungal nourishment. This conversion is brought about by the chemical action of enzymes secreted by the vegetative strands, or hyphae, which ramify through the wood, penetrating and eventually destroying the cell walls. In the early stages of hyphal attack, evidence of chemical corrosion is sometimes observed in advance of actual piercing of the cell wall, but, in general, the hyphae must penetrate cell after cell to obtain sufficient food for the growing fungus.

The first investigators in the field of forest pathology recognized and recorded the penetration of the cell walls of wood by the hyphae of xylophagous fungi, and the fact that such penetration was accomplished in part, at least, by chemical means was established at an early date. As a result of the confidence in these conceptions, the holes made by hyphae have long been accepted as a *fait accompli*, and virtually no direct research has been undertaken to establish the facts dealing with such penetration.

Early workers, notably T. Hartig (1833), Unger (1866), Willkomm (1866), and particularly Robert Hartig (1874, 1878, 1894, 1900), accurately depicted hyphae passing completely through the walls of wood cells, but they made no reference to the preliminary stages of the penetration. It is significant that Hubert (1924: 545), referring to the work of Robert Hartig, states that, "Since Hartig's time comparatively little has been added to our knowledge of the subject."

The chemical nature of decay has long been recognized. Since the work of Czapek (1899) on the production of enzymes by wood-inhabiting fungi, many enzymes have been isolated and numerous workers have contributed to the general knowledge of this subject. Zeller (1916) reported the presence of catalase, cellulase, diastase, emulsin, esterase, hemicellulase, hippuricase, inulase, invertase, ligninase, maltase, pectinase, protease, raffinase, rennet, tannase, and urease in mycelial mats of *Lenzites saepiaria* Fries. Schmitz and Zeller (1919) and Schmitz (1919, 1920, 1921), in working with six other representative wood-destroying fungi, confirmed the presence of the enzymes previously reported by Zeller and added amidase and lactase to the list. Mayo (1925) contributed

glycogenase, oxidase, reductase, and tyrosinase in his work with *Stereum purpureum* Pers. A number of other investigators have demonstrated the presence of various of these enzymes in the mycelial mats of other xylophagous fungi.

The discoveries by these workers, together with concurrent progress in related chemical fields, has established the chemical nature of decay and the gross part played by the enzymes in the process. Yet, while the existence of enzymes capable of attacking the ligno-cellulose complex has been established, little is known of the action of individual enzymes in their attack on wood. The end products of decay, the result of total enzymatic activity, are fairly well understood, but the fact that a certain fungus causes a brown rot and that another produces a typical white rot does not throw any direct light on the manner in which specific enzymes are brought into play in cell-wall penetration. In the brown rots the general enzymic attack is against cellulose whereas in the typical white rots the causal fungi exhibit a preferential attack on lignin, yet in both cases penetrating hyphae are able to dissolve both cellulose and lignin in their passage through the cell wall. This is illustrated in Plate XXI which shows a hypha of a brown-rot fungus traversing the longitudinal plane of a middle lamella.

Nothing is known about the sequence of the production of, or the attack by, enzymes in the course of the decay process. The fact that some fungi lack certain enzymes has been established, but the reasons, in most cases, are not understood.[1] There is the possibility that synergies between enzymes may play a part, and, in the process of penetration, there may be functional as well as qualitative secretions of enzymes. There may even be a single enzyme capable of dissolving both lignin and cellulose. Also, as suggested by Campbell (1930), some of the by-products of enzymic action may catalyze subsequent reactions. Not the least of the unknown factors is our incomplete knowledge of the chemical nature and the micro-structure of the wood complex. Thus, for the purposes of this investigation, it has been necessary to deal only with the total results of enzymatic activity rather than with the specific action of any particular enzymes.

In spite of all these uncertainties, the known presence of enzymes

1. It is well known that some fungi are narrowly specific to certain groups of woods while others are catholic in their attack. The fact that fungi do not all produce the same enzymes suggests the possibility that specific attack may have its origin in a correlation between the enzymes present and the chemical composition of the wood attacked.

capable of dissolving cell-wall substances has resulted in a general acceptance of the chemical concept of penetration by the hyphae of xylophagous fungi, but the facts have been assumed rather than substantiated by concrete evidence.

At the same time, the possibility that mechanical force may be involved is often expressed by pathologists, although the statement is rare in print. This belief has probably evolved from the fact that the presence of mechanical force has been clearly demonstrated in the penetration of the soft cellulosic tissues of living plants by parasitic fungi (Brown and Harvey, 1927). Hubert (1924: 545), in commenting on the possible extension of this concept to the penetration of lignified walls, says, "The theory of enzyme action more nearly fits the facts observed."

Cartwright (1930), in an excellent paper on the decay of Sitka spruce by *Trametes serialis* Fr., has suggested the hypothesis that mechanical force may be exerted after enzymatic activity has produced a general softening of the adjacent wood. Boyce (1938: 382) minimizes the probability that it can be an important factor.

Most species of wood-destroying fungi produce at least two types, or sizes, of hyphae (Plates XI and XII). In general, the small, actively growing hyphae penetrate the cell walls without appreciable reductions in diameter (Plate IX), whereas, in the early stages of decay, the larger hyphae of the same fungus (Plates II and XIII) usually become constricted on contact with the cell wall and effect the passage at a reduced diameter, with the result that the bore holes produced by the two sizes of hyphae are often of approximately the same diameter. Cartwright (1930) described the attenuation or constriction of such hyphae just previous to contact with the cell wall and based his suggested hypothesis on this observation. The awl-like configuration of the hyphal tips undoubtedly inspired or influenced his decision that mechanical force may be an instrumentality of penetration.

Fungi may also utilize the natural inter-communications between cells, such as the simple and bordered pit-pairs in the cell walls (Plates XIII, XIV, and XV). This is known to occur among the well-known staining fungi, but the penetration in this case takes place in the sapwood while the closing membranes of the pits are still soft and pliable. After sapwood has been transformed into heartwood or after it has partially dried, as is the usual condition of wood attacked by wood-destroying fungi, the membranes of the pits offer essentially the same resistance to penetration as do the cell walls. This statement is confirmed by Plate

XIII in which the hypha is seen to have undergone the sharp constriction that usually precedes the penetration of a solid wall. Wood-destroying fungi do not specifically seek out pit-pairs as passages through wood (Plate XIV), and, although hyphae are very commonly observed using these openings, the process can be dismissed insofar as it represents a distinctive method of penetration.

THE PROBLEM

THE object of this investigation was to determine the manner in which the hyphae of wood-destroying fungi penetrate the cell walls, whether by chemical action or mechanical force or by the concurrent action of both agencies.

Essentially, the problem embraced the following steps:

1. The preparation of decayed wood by the inoculation of wood blocks with pure cultures of a suitable number of representative fungi.

2. The sectioning of the wood blocks at the proper stage of decay and the preparation of slides for observation under the compound microscope.

3. A careful and systematic search of all material for any and all evidential facts, either direct or indirect, bearing upon the mechanisms of penetration.

4. Perpetuation of the observations in the form of photomicrographs.

It is difficult to obtain thin sections (2–6 microns) of decayed wood because the decay processes weaken or destroy the wood structure. Also, the preparation of a microtome knife for the purpose of securing such sections requires inordinately long periods of sharpening in order to maintain the critical edge that is an absolute necessity for this type of work.

The hyphae of many wood-destroyers are microscopic in size, often less than one micron in diameter (Plate XI), and, more often than not, completely hyaline; a combination of conditions which makes their detection extremely difficult. This imposes the necessity of employing differential stains, and the uncertainties connected with staining fungi in wood are patent to all histologists who have attempted it. Most of the classical methods of staining are, for one reason or another, inadequate. The most obvious objections are poor differentiation of color as between the wood and the fungus, and unnatural shrinkage and distortion of the hyphae. The latter can be caused by the severity of the stain itself, its concomitant mordants, or the use of excessive heat in the method of application.

Most of the traditional mounting media also possess certain undesirable features, the most important being the necessity of dehydration of the material and the consequent shrinkage and hardening of the hyphae. The inability to secure satisfactory color differentiation by staining also introduces serious obstacles to the attainment of sufficiently critical photomicrographs.

The success of the entire investigation depended on the finding of hyphae in the actual act of penetration. Cartwright (1930: 8) calls attention to the difficulty of this important aspect of the problem in saying, "It was notable that, although a diligent search was made for early stages of penetration, no definite case of this was seen . . . ." This statement is indicative of the amount of material, and searching of sections, that was required.

EXPERIMENTAL TECHNIQUE

Inoculations

SIX species of fungi were used in the investigation, namely: *Fomes annosus* (Fr.) Cke., *Fomes pini* (Thore) Lloyd, *Lenzites trabea* (Pers.) Fr., *Polyporus schweinitzii* Fr., *Poria weirii* Murr., and *Trametes serialis* Fr. Inoculations were made on four woods: eastern white pine (*Pinus strobus* L.), western hemlock (*Tsuga heterophylla* [Raf.] Sarg.), Douglas fir (*Pseudotsuga taxifolia* [Lam.] Britt.), and western red cedar (*Thuja plicata* D. Don). Wood of red maple (*Acer rubrum* L.) was inoculated with *Stereum purpureum* Pers. for use in staining experiments. It is believed that this number of fungi and woods was sufficient to give a representative cross section of the conditions prevailing in the decay of wood by xylophagous fungi. The pertinent information concerning the inoculations is presented in Table I.

The pure cultures used were kindly supplied by the Forest Products Laboratory, United States Forest Service, Madison, Wisconsin, and by the Division of Botany and Plant Pathology, Department of Agriculture, Ottawa, Canada; the woods by Dr. George A. Garratt, Professor of Lumbering, Yale School of Forestry, and Dr. Clarence D. Stone, Assistant Professor of Wood Technology, University of Idaho.

Before culturing the blocks, sections were cut from several samples of each board used and these were carefully scrutinized for any signs of decay. As an added precaution, controls were established for each species of wood by incubating sample blocks on un-inoculated agar.

Preparation of the Culture Blocks

Pre-cut culture blocks were used (Plate I). These blocks were made from blanks 1 inch in cross section by 1½ inches long. They were then cut on a band saw (or with a small hack saw) in such a manner that a small boss about $\frac{15}{16}$ inch in cross section and ¼ inch long projected from each side of the block. Such blocks have an important advantage. The projecting bosses are carefully trimmed to expose *true* radial, tangential, and transverse faces before the wood is inoculated. In consequence, the

TABLE 1. FACTUAL DATA PERTAINING TO WOOD CULTURES

Fungus	*Source*	*Wood*[1]	*No. of cultures*	*Time in culture. Days*	*No. of slides made*
Trametes serialis	U. of W.[2] (originally from F.P.L.)	Western hemlock	15	73	70
		Douglas fir	8	69	25
Fomes annosus	Canada[3] F-8415	Western hemlock	11	127	60
		Douglas fir	8	132	40
		Eastern white pine	10	70	25
Fomes pini	F.P.L.[4] 639	Western hemlock	14	77	47
		Douglas fir	15	112	30
		Eastern White pine	10	123	27
Polyporus schweinitzii	Canada F-8284	Western hemlock	3	132	35
		Douglas fir	7	125	40
		Eastern white pine	8	134	20
Lenzites trabea	F.P.L. 617	Western hemlock	10	75	40
		Douglas fir	8	112	20
		Eastern white pine	3	107	30
Poria weirii	Canada F-8412	Western red cedar	8	110	38
Totals			138		547

1. Woods obtained from Prof. G. A. Garratt, Yale School of Forestry, and Dr. C. D. Stone, University of Washington, now Asst. Prof. University of Idaho.
2. University of Washington.
3. Department of Agriculture, Ottawa, Canada.
4. Forest Products Laboratory, U.S. Forest Service, Madison, Wisconsin.

blocks can be sectioned, without further trimming, as soon as they are removed from the cultures. This eliminates the splitting, sawing, and trimming to which the wood must be subjected when solid blocks are prepared for sectioning. It results in greatly reduced disturbance of the friable wood elements and the contained fungus.

Wood cultures are usually prepared in the following manner: A suitable growth medium is poured into the culture flask and sterilized. After cooling, the medium is inoculated and the fungus is allowed to develop a luxuriant growth. Separately sterilized blocks are then dropped on to the culture. Contaminations often occur during this operation, and when they do, the time spent in growing the culture is lost. By placing the blocks on an inclined microscope slide (ordinary slides were exactly the proper length for the 4-oz., wide-mouth bottles used, Plate I), the block and the agar medium can be sterilized simultaneously, eliminating one step of the usual procedure. The inoculation is then made after the manner employed in making transfers of slant cultures. This not only reduces the chances of contamination, but should it occur, the cultures can be re-sterilized or discarded before time has been lost in growing the inoculum. The elevation of the block above the agar also permits a more careful check of the progress of decay into the part that is to be sectioned. The use of individual cultures permits periodic removal of blocks without contaminating those remaining.

Blocks of this type are not satisfactory if allowed to reach advanced stages of decay unless they are embedded before sectioning, as otherwise it may be difficult to hold the weakened base in the vise of the microtome. The method was not successful with cultures of *Fomes annosus* or *Poria weirii* because the mycelia of these species remained closely appressed to the agar and failed to bridge the narrow gap between the medium and the wood. In both of these cases it was necessary to remove the microscope slides and drop the blocks into contact with the mycelial mats.

It is realized that the procedure followed does not accurately simulate natural conditions of decay, but it seems tenable to assume that the mechanism of penetration would be comparable, particularly since all blocks were sectioned while the wood was in the early stages of decay.

Media and Methods of Sterilization

While wood blocks can be inoculated directly, a growth medium was used to facilitate the rapid establishment of the fungus and thus expedite

attack of the wood. DIFCO[2] Bacto malt agar was used almost exclusively. Potato agar, manufactured by the same company, was similarly employed in a small number of cultures, but without visible difference in the establishment of the fungi. Both media were prepared, poured, and sterilized in accordance with usual laboratory practice, the sterilization process being conducted in a standard autoclave for twenty minutes at fifteen pounds gauge pressure.

Some pathologists believe that steam sterilization of wood may alter, translocate, or remove certain of its constituents to an extent that may influence hyphal behavior. Changes in the composition or the availability of some of the substances in wood that are used by fungi for food can undoubtedly occur, but whether the effect on hyphal behavior is significant has not been determined. The large number of cultures used made it possible to set up a limited number of matched experiments for a cursory examination of this postulation.

Half of the cultures of *Fomes annosus* on eastern white pine and of *Trametes serialis* on Douglas fir were sterilized by steam, the remainder by acetic acid fumes, as suggested by Fritz (1930), and dropped into culture bottles containing actively growing mycelial mats. Later, blocks from each series, in comparable stages of decay, were sectioned and compared, but no visual differences in hyphal behavior could be detected. From these limited observations it appears that any changes produced as a result of treatment with steam must be manifested in the ultimate chemical nature of decay, rather than in physical modifications of hyphal behavior.

The acetic acid method has certain disadvantages. First, it is time-consuming and requires large amounts of distilled water and rather large glass containers if many cultures are prepared. Second, it sterilizes only the exterior of the blocks and does not prevent the growth or revival of viable hyphae at the interior of the wood. Under some conditions this demands extra precautions in the use of controls.

Cultures prepared in this manner did not become contaminated during the necessary manipulations. Initial attack of the wood was retarded, presumably because of the impossibility of removing all traces of the acetic acid, but after the outer sterilized zone had been penetrated, decay appeared to progress as rapidly as in the steam-sterilized blocks.

The acetic acid method proved useful in the preparation of cultures of

2. DIFCO Laboratories, Inc., Detroit, Michigan.

Poria weirii on western red cedar, the principal host of that fungus, since steam sterilization extracts from the wood certain water soluble substances that are highly toxic to fungi. In fact, it was impossible to induce decay by this fungus on cedar blocks that had been autoclaved. The acetic method was successful, as was also direct flaming of the blocks with absolute alcohol. Flamed blocks are reported by Fritz (1930) to be more subject to contamination, but cultures so treated remained pure and the fungus attacked the wood much more rapidly than was the case with blocks sterilized with acetic acid fumes.

The blocks to be flamed were soaked for several hours in sterile water and then flamed three successive times in absolute alcohol. The last flaming had a tendency to char the edges of the blocks, but this was of no consequence. The method possesses the same disadvantage as the acetic acid method with regard to internal sterilization, but it is much more rapid and the results appear to be equally as good, if not superior. Both of these methods are designed to provide surface sterilization with the object of preventing accidental surface contamination. If successfully accomplished, this should be sufficient, since blocks containing internal infection, even if killed by complete sterilization, should not be used in pure culture experiments.

Storage and Growth of Cultures

All cultures were stored in a tightly sealed glass cabinet which was exposed to the normal daily light of a room with southwest exposure. Temperatures varied within the relatively narrow limits of 67° F. to 72° F. The relative humidity of the air in the cabinet, as measured by a hair hygrograph, was maintained between 95 and 100 per cent by providing a large exposed surface of water. All cultures exhibited satisfactory growth under these conditions and all produced early or intermediate stages of decay in less than four months. *Trametes serialis*, *Lenzites trabea*, and *Fomes pini* displayed luxuriant growth in less than one month, and the first two, in particular, can be recommended for any studies in which time is an important factor. Both also produced sporophores in culture, as did *Polyporus schweinitzii*. The sporophores of *Lenzites trabea* were poroid when first formed, whereas the mature fructifications are normally lamellate.

One experimental set of inoculations failed to produce decay. Western red cedar, a highly durable wood, was inoculated with *Trametes serialis* and *Lenzites trabea* but neither attacked the wood. In fact, soluble ex-

tractives that were leached from the wood by moisture contained in the agar finally resulted in killing the fungi.

Sections

Sound wood of relatively uniform texture is not difficult to section with a sliding microtome. Decayed woods, and woods with a soft or porous earlywood and a sharply delimited, dense band of latewood, on the other hand, often shatter and tear when cut. This is usually obviated by embedding a block in a matrix of celloidin which maintains the various structures in their relative positions while the sections are cut. Standard embedding methods require the complete dehydration of the materials to be infiltrated and the application of heat to insure penetration of the sustentive matrix. As previously stated, such treatments tend to induce unnatural shrinkage, distortion, and hardening of delicate structures. Also, in cutting thin sections, the celloidin which accumulates in the cells is likely to buckle and to be pushed along in front of the knife, thereby seriously disturbing the hyphae lying loosely packed in the cell cavities. Embedding was, therefore, so far as possible avoided and the difficulties of sectioning untreated wood were tolerated in the interest of preserving natural conditions. Such sections may contain broken or torn areas, but good parts large enough for observation can usually be found. All non-embedded blocks were soaked in sterile water for several hours immediately on removal from the cultures and then cut, using a 2 per cent solution of Aerosol OT Clear[3] in distilled water as a lubricant for the microtome knife.

Douglas fir, which is a coarse wood, was the only one embedded. The use of heat was avoided by forcing the infiltration of the celloidin by means of twenty pounds of air pressure in an ordinary pressure cooker with an automobile tire valve. Pressure was easily maintained and evaporation of the celloidin solvent eliminated by placing an open petri dish of ether beside the open vials containing the material being embedded. The celloidin was removed and the sections hydrated through successive alcohols.

One small block of Douglas fir wood which was rather badly decayed by *Polyporus schweinitzii* was embedded by the butyl alcohol-paraffin method. It is often stated that paraffin matrices are not sufficiently firm

3. A wetting agent manufactured by the American Cyanamid and Chemical Corporation, New York, N.Y.

to be used for woody tissues, but sections cut from this block were infinitely superior to those obtained from the blocks embedded in celloidin. Sections as thin as two microns were obtained and the hyphae in the cell lumina were less seriously disturbed. On the basis of this limited trial, the method appears to possess definite possibilities for use where embedding cannot be avoided. A further advantage is derived from the possibility of affixing sections permanently to microscope slides as is done with paraffin ribbons cut on a rotary microtome. This eliminates further handling of the sections during staining and mounting.

Sections cut for observation were, for the most part, either six or eight microns thick. A few were cut at two to four microns for examinations of actual penetration but it was found that extremely thin sections were unnecessary unless minute detail of this nature was to be studied. Sections of at least twelve microns are desirable for observations of the distribution of hyphae, but the photography of isolated hyphae or other individual features is difficult against a solid background of wood. Radial sections were relied upon as a source of most of the observations because they are more easily cut and because fungi are seen to better advantage in this plane. However, features found in tangential sections were also photographed.

Staining

The number of stains that can be used to stain fungi is strictly circumscribed. Nearly all of the stains that are successful in coloring isolated fungal material are ineffectual as counter stains for fungi in wood. The conditions dictated by the problem at hand imposed an added curtailment of choice. Since photomicrographs were to be taken with ultra violet light the sections were mounted in a medium adaptable to this technique, and the medium chosen was water soluble. Thus the stains were required to resist leaching by water, at least for several weeks. As it was also desired to avoid shrinkage and distortion, by whatever means caused, stains depending upon dehydration, the application of excessive heat, or possessing any other inherently undesirable features had to be eliminated.

Most of the staining schedules devised for the purpose contain one or more of these limiting factors, or they are excessively laborious or slow. A proprietary dye used in the home dyeing of ordinary fabrics was found to be remarkably specific to the fungus used in a preceding investigation, namely *Trametes serialis*. On continuing the staining experiments in the

present work it was found that the stain was not effective for all fungi in all woods and efforts were made to find a mordant that might accomplish the desired ends. In the meantime, the color of the reserve supply of the dye, which was prepared in paste form, had deteriorated from a bright cardinal red to a dirty brown, and efforts at replacement disclosed the fact that the manufacturer had withdrawn from business. Through the good offices of the Dyestuffs Division, Organic Chemicals Department, E. I. du Pont de Nemours & Company, it was found that the dye was very similar to their product "Pontamine" pink 2B[4]. This proved to be almost as effective as the original product, but was subject to the same limitations in staining some of the other fungi.

Efforts to improve the staining qualities of the dye disclosed that a solution made up of one part saturated aqueous solution of "Pontamine" pink, one part saturated aqueous solution of picric acid, and four parts of distilled water resulted in a distinct improvement. However, even then it was only partially effective, being limited in its usefulness to *Trametes serialis* and *Lenzites trabea*, and in lesser degree to *Poria weirii*. The entire schedule follows:

1. Stain for one or two minutes in a 0.01 per cent aqueous solution of malachite green (about one drop of 1 per cent aqueous malachite green to 10 cc. of distilled water). This stains the wood elements.

2. Stain 10–20 minutes in picro-pontamine pink of the strength described above. This stains the hyphae a pale, but clear, red color.

3. Wash in distilled water and mount in Karo mounting medium. (See "Mounting Media," next section.)

Sections stained by this method should be photographed within one or two weeks, since the stain sometimes leaches from the hyphae, although this behavior is not constant. This technique is admittedly imperfect, but it was used at every opportunity because of its superior differentiation in photomicrographs taken with ultra violet light. When successful, the red dye is strikingly specific and it is hoped that someone will succeed in extending its utility.

Because of the failure of the above stain to have universal application it was necessary to perfect another method. Of all the standard methods, Cartwright's (1930: 21) picro-aniline blue is by far the most positive and

4. This class of dyes appears to have some promise in the field of biological stains. The du Pont Company has expressed a willingness to supply this, or similar dyes, through the usual agencies dealing in biological stains.

it appeared to offer possibilities for the problem at hand since both of the stains used are applied in aqueous solution. Picro-aniline blue is prepared by mixing one part of saturated aqueous aniline blue with four parts of saturated aqueous picric acid. Cartwright's schedule is here given so that it may be compared with the schedule finally adopted.

1. Overstain sections in 1 per cent aqueous safranine.
2. Wash in distilled water, but be sure to leave sections overstained.
3. Place a section on a microscope slide and flood with one or two drops of picro-aniline blue.
4. Heat over a flame until the stain commences to simmer.
5. Wash.
6. Dehydrate rapidly, clear, and mount in balsam.

The wood is stained red, the hyphae blue.

While this technique will invariably demonstrate the presence of hyphae in wood, it embodies several objectionable features, the most serious resulting from the excessive heat to which the material is subjected. Sections heated in this manner usually shrivel or become distorted, and comparative examinations of heated and unheated sections showed that the hyphae suffer the same fate. Also, the heat causes granular masses of aniline blue to be precipitated on the hyphae. This, combined with the intense staining obtained, tends to obscure structural details. While dense staining is often required for photography with visible light, it is not desirable with ultra violet light.

Empirical experiments disclosed that very dilute concentrations of picro-aniline blue were equally effective in staining the fungi, providing weaker safranine solutions were also used. Heat was found to be unnecessary with sections that were to be photographed within a few days, but a reduced amount of heat proved effective in making the stain more permanent. The schedule adopted follows:

1. Stain sections 1–3 minutes in 0.01–0.02 per cent aqueous safranine (about 1–2 drops of 1 per cent aqueous safranine in 10 cc. of distilled water).
2. Wash thoroughly in distilled water.
3. Place any desired number of sections in a small beaker and cover with 10–20 cc. of a 0.01–0.005 per cent solution of picro-aniline blue (about 1–½ drop of full-strength picro-aniline blue in 10 cc. of distilled water). Stain for 10–20 minutes without heating; or heat gently over a micro-burner for 2–3 minutes at not to exceed 45–50° C.
4. Wash in distilled water and mount in Karo mounting medium.

This schedule produces sharp color contrast in combination with clear, translucent sections which can be photographed with ultra violet light. However, the absorption of ultra violet light by the two stains is so nearly equal that the results are not as satisfactory as those obtained with the picro-pontamine pink. The principal advantage lies in its general adaptability to the staining of fungal hyphae in wood. The above schedule makes rapid staining possible and it is well adapted to staining a large number of sections at one time. By removing and adding solutions with a syringe pipette the entire operation can be conducted in one vessel, thereby reducing handling of the sections to a minimum.

Several cautions should be considered. Some mycelia include a type of hyphae having dense, thick walls and no apparent contents. These usually accept the counter stain used to color the wood and any attempts to stain them with the fungal stain usually result in serious overstaining of other hyphae. Ordinarily they can be photographed without special staining.

Adjustments of the schedule are often required for different fungi and different woods, or for the same fungus in different woods. When staining many sections at one time it is advisable to run one or two of them through the schedule to gauge any changes that may be required. The schedule given applies to mounting in a water soluble medium, hence further adaptations may be necessary if the material is to be dehydrated.

Distilled water should always be used throughout the schedules to preclude the possibility of introducing extraneous spores and other contaminants.

The extreme dilution of the stains may cause concern to those accustomed to the usual schedules, but the reason will be obvious when the subject of photomicrographs is discussed in a later section.

Mounting Media

A mounting medium to be used successfully with the technique employed was required to eliminate the necessity of dehydration and, at the same time, to possess those properties required in ultra violet photomicrography. A 50 per cent solution of Karo[5] corn syrup in distilled water had previously been found to satisfy these requirements. The use of Karo as a mounting medium was first reported by Patrick (1936), but Professor Bror L. Grondal, of the College of Forestry, University of

5. Red Label Karo, Corn Products Refining Company, Argo, Ill.

Washington, can probably be credited as being the first to use it in connection with ultra violet photography.

This medium has a number of advantages. The necessity of dehydration, a prerequisite to mounting in Canada balsam, is obviated. Stained sections can be mounted with great rapidity. Sections to be mounted are covered (in a Syracuse watch glass) with 8–10 cc. of the 50 per aqueous cent solution of Karo. Each section is then lifted out with a small camel hair brush and placed directly on the microscope slide and the cover glass put in place. The small quantity of Karo that adheres to the brush and the section is just sufficient to insure a neat mount. Bubbles can be avoided if a small drop of Karo is placed in the center of the cover glass and allowed to fuse with that already on the slide before dropping the cover glass into its final position.

The mounts harden in the same manner as balsam preparations, but more rapidly, and require no ringing. They can be examined in ten to fifteen minutes without fear of displacing the cover glass, and after several hours of drying they can be photographed, even with oil immersion objectives.

The time required to mount the sections is only slightly longer than would be required to mount in water. Consequently, rapid examinations can be made to eliminate unsuitable material, and the discarded slides may be returned to service by simply rinsing them in hot water. If strictly permanent mounts are desired of sections that have already been mounted, the Karo can be readily dissolved by water and the sections then washed, dehydrated, cleared, and mounted in balsam.

The Karo, being largely a sugar solution, undoubtedly exerts a high osmotic pressure. Nevertheless, it does not appear to plasmolyze the hyphal cells to any marked degree. Serial sections mounted in Karo and water, respectively, displayed no visible differences.

Canada balsam has a relative absorption of the 3650 Ångström unit line of ultra violet light of 82 (Eastman Kodak Co., 1935: 68), as compared to virtual transparency for glycerine. It is, therefore, not well adapted to photography with this wave length, although presentable photomicrographs can be made of sections mounted in this medium if suitable exposure allowances are made. Either glycerine or mineral oil are suitable, but both of these media require ringing of the cover glass to prevent their drying out. At best, ringing is time-consuming. Further, it is very difficult to secure perfectly flat sections of wood in ringed mounts and the liquid condition of the medium often permits slight movements

of the section. Both of these features are serious faults in slides to be used for photomicrographs with oil immersion objectives. While the relative absorption of Karo is not known, tests show that it compares very favorably with glycerine and is, therefore, an ideal mounting medium for the purpose under discussion.

Karo mounts are fairly permanent in regions of low summer humidities, and four-year old slides kept under these conditions are still intact. In regions of hot, humid summers they may be attacked by molds if not ringed or stored in a desiccator during the unfavorable months. Precautions should always be taken to prevent contamination of the supply bottle of Karo, otherwise it is possible to introduce artifacts into the sections. Thymol is a suitable antiseptic, but it tends to turn the Karo milky. This can be avoided by suspending a small cloth bag filled with thymol crystals from the cork with which the flask is stoppered.

Ultra Violet Photomicrography

Successful photomicrography with visible light depends upon brilliant, clear-cut differential staining. Since this is difficult to achieve with fungi in wood, ordinary photographic methods give, for the most part, only mediocre results. For this reason ultra violet photomicrography was chosen as the best means of securing visual records of pertinent observations.

Ultra violet photomicrography of the past has utilized the 2750 A° line from a cadmium arc for illumination. Since this wave length is totally absorbed by even optical glass, expensive equipment in the form of a complete quartz optical system and a special monochromator must be used. The technical manipulations are also complicated, particularly since it is necessary to focus an invisible object. This can be accomplished, in most cases, only by empirical exposures.

About 1930 the Eastman Kodak Company and the Bausch & Lomb company coöperated to develop a compromise which would retain most of the benefits of using the shorter wave lengths and, at the same time, eliminate the serious objections to the older methods. The basic concept is the utilization of ultra violet light having a wave length just sufficient to avoid serious absorption by optical glass (Eastman Kodak Company, 1935; Trivelli and Foster, 1931). The result was photomicrography with the 3650 A° spectral line.

The Bausch & Lomb Company has computed specially corrected glass objectives for this purpose with which it is possible to focus an

object in exactly the same plane in both the 3650 A° line and a visible green light with a wave length of 5460 A°. This is accomplished by the use of two filters in connection with a single light source. The distinct improvement over the older method arises from the fact that it is possible to focus visually in the green light. A list of the equipment required is supplied by the Bausch & Lomb Company, Rochester, N.Y., but the essential parts will be enumerated.

A special high-intensity light source is manufactured for use with the special optics, but the modern tungsten-arc mercury vapor lamps used in ordinary photomicrography supply ample ultra violet radiation of the desired wave length when fitted with a quartz condenser. The quartz Abbe condenser and the special objectives are less expensive than corresponding apochromatic optics. Ordinary compensating eye pieces are used. Corex slides, which are expensive, are sometimes recommended, but are unnecessary if clear water-white microscope slides of good quality are selected.

Sandalwood oil with a relative absorption of the 3650 A° line of 32 is used with the oil-immersion objective in preference to the usual cedarwood oil which has a relative absorption of 56. As previously stated, glycerine, mineral oil, or sugar solutions are superior to the customary mounting media, although the latter may be used.

Any laboratory possessing a good microscope, a suitable light source, and a set of compensating eye pieces can add ultra violet equipment at a very nominal cost.

Ultra violet photomicrography with this equipment offers at least two important advantages. As all microscopists know, the resolving power of a microscope using visible light cannot be increased beyond certain practical limits which are fixed by definite optical principles. Of the factors involved, only a reduction in the wave length of the illumination used can bring about an increase in resolution after these optical limits have been reached. By thus reducing the wave length through the use of the 3650 A° line, resolution is improved from 12 to 19 per cent, and higher magnifications without loss of detail and better definition at lower magnifications are obtained. The significance of this advantage is evident when the size of the subjects studied is considered.

An equally important advantage results from the fact that many biological materials exhibit differential absorption of the 3650 A° light. This often obviates the necessity of staining, with the result that much detail that might otherwise be obscured by the stain can be photographed. Un-

fortunately, this advantage could not be fully capitalized because of two conditions which could not be avoided. First, the walls of many of the hyphae appeared to have approximately the same absorption as those of the wood cells. Secondly, the extreme difficulty of locating the smaller hyphae for examination made staining an absolute necessity. However, this inherent superiority of the method was preserved to some extent by the use of the dilute stains previously described.

The steps followed in taking pictures with this equipment are identical with those of the usual method, with one slight exception. After the subject to be photographed has been found with visible light, the green filter is placed in front of the lamp and the subject focused. The iris diaphragm is then placed at the desired opening, the green filter removed, the ultra violet filter inserted, and the film exposed. The difference lies in the slightly increased difficulty of focusing in the reduced green light. This must be done in darkness or under a black cloth. Exposures are somewhat longer, but experience is acquired very easily by the use of a few exposure-test negatives.

The advent of the electron microscope and the high magnifications of which it is capable has had a tendency to minimize the value of the ultra violet technique in the biological sciences. In its present stage of development the electron microscope can be employed successfully only with objects one micron or less in thickness. In fact, one micron approaches the upper limit. It is of inestimable value in studying individual details of minute structures, but as yet it is not adapted to the study of sectioned materials. Until this is made possible the ultra violet method will still have wide application in the investigation of plant and animal tissues.

Photographic Technique

Sections searched for subjects to be photographed were examined under the microscope used in the photomicrographic camera. Each slide received a distinguishing number as examined and the position of each detail was recorded by means of the calibrations on the mechanical stage. These numbers were accompanied by a rough sketch to aid in distinguishing the desired detail and to facilitate identification of the negatives.

Ordinary process film was used. The Eastman Kodak Company recommends orthochromatic film for this purpose, but increased speed is the only gain since chromatic correction is unnecessary at the short wave lengths used. The advantage of being able to load film holders and develop in yellow light more than offsets the slightly longer exposures that

are required. Process film gives a good black and white contrast which tends to eliminate the gray monotones that are characteristic of many photomicrographs.

After a few experiments had been conducted with other developers, films were processed by the tank method with Eastman Kodak DK 20 fine grain developer. It produces very satisfactory negatives and, because of the fine grain feature, contact lantern slides made from the negatives project very well. Contact prints were made on standard Azo papers of varying degrees of contrast and ferrotyped to give a high gloss.

Magnifications Used

The high magnifications recorded may elicit some comment, but in nearly every case the lowest magnification that would bring out the desired detail was used. With some subjects the enlargement of low-power photomicrographs gives the best results. However, when minute structural details are photographed in an object of much greater thickness (such as hyphae one micron in diameter embedded in a wood section eight microns thick) the surrounding structures are usually out of focus and the resulting negatives have a tendency to be rather dense and "fuzzy." This condition mitigates against the thin, clear negatives required for enlarging and, with but few exceptions, enlargement added nothing to the quality of the photographs.

Magnifications of 500, 750, 1000, 1500, 2000, and 2500 diameters were obtained with the 1.7 mm., 1.3 N.A. oil immersion objective. With the camera used, a maximum magnification of 4000× was possible. With the bellows extensions used, the 15× eye piece gave magnifications of 2500×, 2000×, and 1500×; the 12.5× eye piece, 1500× and 1000×; the 7.5× eye piece, 1000× and 750×; and the 5× eye piece, 750× and 500×. All eye pieces were of the compensating type. One photograph included in the illustrations (Plate XX) was taken with the 6 mm., 0.65 N.A. dry objective and the 15× eye piece at a magnification of 350×.

All magnifications were verified by making a separate ultra violet exposure of a substage micrometer, with each combination of optics, and carefully measuring the calibrations.

Observation of Bore Holes with Polarized Light

Two hundred bore holes (in both cross and longitudinal sections representing all of the fungi studied) were observed under polarized light for any traces of mechanical failures that might have been caused by the

application of mechanical force by the hyphae that formed them. Polaroid discs were used and part of the observations were made with the intense light of the tungsten-arc lamp. Not a single bore hole displayed interference patterns of any kind that might indicate the presence of mechanical failures.

During the entire investigation literally hundreds of bore holes were examined with meticulous care for the slightest evidence that mechanical force might have played a part in their formation. Every bore hole exhibited the unmistakable stamp of chemical corrosion as expressed by the smooth, molded contours of all surfaces and the circular form which, in itself, is indicative of the dissolution caused by a solvent having its origin at a central point. Of all the bore holes examined, none was jagged, torn, or splintered.

Spirally oriented cracks (Plate XXII) which are sometimes found in decayed wood, often pass through or near bore holes, but they are usually of such size that it is inconceivable that they could be caused by the mechanical action of boring hyphae. The extended apertures of bordered pits (Plate XVIII) sometimes have a similar appearance. Compression failures of the cell walls also occur at bore holes, particularly in woods decayed by *Fomes pini*, *Poria weirii*, and other fungi in which numerous hyphae traverse the cells at right angles to their longitudinal axes. These too, however, are effects, not causes. They result from the presence of many contiguous bore holes and are not caused by the hyphae at the time the cell walls are penetrated.

It may be remarked, in passing, that the term "bore hole" implies the application of rotary mechanical force in the perforation of a resistant substance. All of the evidence adduced by this investigation indicates that its use in connection with the penetration of a cell wall by a fungus is inappropriate, but the term has become too firmly entrenched in pathological terminology to be easily replaced.

DISCUSSION

A REVIEW of the methods of penetration that may be employed by fungi in attacking plant tissues discloses that only two may be used by xylophagous fungi in penetrating the walls of wood cells. These are: (1) penetration by the secretion of chemical substances by the hyphae and the total dissolution of the wall constituents in advance of actual passage, and (2) the application of a small amount of direct mechanical

force after chemical action has dissolved, or softened, the constituents of the cell wall. In the absence of supporting evidence of any kind, penetration by direct mechanical force alone can be dismissed as untenable.

An ideal solution of the problem at hand would provide positive proof that one of these instrumentalities is entirely responsible for penetration, and conclusive evidence that the other is not. The findings of this investigation will, it is believed, establish the positive facts, but the evidence in the negative, while convincing, may not be conclusive.

Cartwright (1930: 8) states that the first step in penetration is a V-shaped notch cut in the cell wall at the point of contact of an advancing hypha. These notches are often observed, but the hyphae forming them are seldom in situ, due largely to the fact that they lie in the open cell lumina and are usually displaced or disturbed in sectioning. This condition is, however, shown in Plate II, where the constricted tip of a relatively large hypha can be seen occupying the notch formed in the thick secondary wall at the end of a tracheid.

This preliminary attack upon the cell wall may be caused by: (1) small hyphae exhibiting little or no diminution in diameter, or (2) larger hyphae whose apexes have become constricted or attenuated into relatively fine points of considerably smaller diameter than the main hyphae. The bore holes produced by the two sizes of hyphae are usually of about the same diameter, although occasionally the constriction of the tip of a large hypha appears to have been delayed, with the result that the initial notch may be somewhat larger than required for the passage of the penetrating hypha. Nevertheless, the processes of penetration are the same.

At the time of first contact with the cell wall (particularly in the early stages of decay before general chemical action is apparent) it seems tenable to assume that the thick, lignified cell wall is reasonably sound and capable of offering resistance to mechanical force of the magnitude that might conceivably be exerted by an instrument as delicate as a fine hyphal strand. Chemical action must, therefore, be initiated at this point, regardless of the ultimate mechanism of penetration that may be employed. It seems likely that actual contact with the cell wall may be the conditioning stimulus which initiates the secretion of enzymes.

By the chemical hypothesis it is assumed that total dissolution of the cell wall is effected in advance of the penetrating hyphae. Proof of this would depend on the presence of an opening or cavity in advance of every hypha observed in the act of penetration, and this fact is clearly demon-

strated in every photograph submitted in evidence (Plates II to IX, inclusive). By this theory it would also be logical to expect that the bore holes would be slightly larger in diameter than the contained hyphae, since it would seem illogical to assume that diffusion of the enzymes could be strictly limited to the immediate region in advance of the growing tip. This contention is also supported by the photographs since even the smallest hyphae were observed to produce bore holes through which they could readily pass; although high magnifications were required to verify and record this fact (Plates IX and XI).

In the theory of partial mechanical penetration, the initial secretion of enzymes at the time of contact is also assumed but the alteration or dissolution of the cell wall is believed to be less specifically directed in the path of the advancing hypha. Cartwright (1930: 8 and 9) states: "The smoothness of the sides of the bore-holes is difficult to account for, except on the supposition that the cellulose of the wall is altered or taken up by the fungus in advance, and that the actual penetration by the fine hyphal tip is thus made easy; this latter process being, at any rate, partially mechanical." This statement seems somewhat contradictory, since, if the wall is "taken up" by the fungus in advance, the necessity for mechanical penetration would seem to be obviated.

If passage through the softened wood were accomplished by mechanical pressure, even in part, the bore holes would not be likely to exceed the diameters of the hyphae at the extreme tip, even though it is possible that they could enlarge immediately after this stage by means of lateral secretions of enzymes. Photographs of penetrating hyphae should, therefore, show: (1) hyphal tips firmly appressed to the extremities of bore holes, and (2) in most cases of incomplete penetration found in early stages of decay (*i.e.*, before lateral expansion of the bore holes has occurred) the bore holes should be no larger than the hyphae which have just forced a passage. In no case was either of these conditions observed. Further, the V-shaped notch of the first stage, which is, in reality, a cone when seen in three dimensions, seems better to fit the case for chemical, rather than mechanical penetration, since: (1) the conical form is exactly that which would be expected to result from the secretion of an enzyme from the peripheral surface of a pointed hyphal tip, whereas (2) the application of sufficient force to effect the penetration of even a yielding substance would tend to produce an irregular indentation, rather than an isometrical, more or less sharply delimited, cone-shaped cavity.

The structure of the secondary wall of woody cells is not completely

understood, although the gross structural elements have been identified by Ritter (1934) and his associates and other investigators. In the above citation Ritter gives a brief but thorough résumé of the important work on this subject up to that date. Professor I. W. Bailey and his associates (Bailey and Vestal, 1937a; Bailey and Kerr, 1937) have confirmed the findings of Ritter.

The work of these investigators has disclosed that the secondary walls of wood fibers are made up of concentric, sleeve-like lamellae, which, in turn, are composed of parallel fibrillous elements called fibrils. The latter are oriented in the general plane of the longitudinal axis of the fiber, although the angular orientation usually varies considerably in the different layers. The fibrils are composed of cellulose micelles. Both the layers of fibrils and the fibrils themselves are believed by Ritter (1934) to be cemented together by substances of ligneous or hemicellulosic origin. The middle lamella, which lies between the secondary walls of contiguous cells, is composed largely of lignin.

Thus, since the various structural components of the cell wall are dissimilar, they are differentially attacked by chemicals (Ritter, 1934; Bailey and Kerr, 1937). In fact, the chemical action of fungal enzymes often reveals the structure of the cell wall (Bailey and Vestal, 1937b). (See Plate XVII.)

Under the mechanical force theory, penetrating hyphae would be confronted with the necessity of forcing a passage through these fibrillose layers before enzymatic activity had reduced the fibrils and intervening substances to an amorphous state. If hyphae possessed sufficient penetrance to force their way through such a substance, it would seem likely that they would produce angular or irregular bore holes as a result of shearing, splitting, and separation of the fibrils and other structural units of the secondary wall. The type of hole that might be expected could be simulated by forcing a small instrument through a very thin sheet of laminated plywood. The examination of hundreds of bore holes failed to disclose anything of this nature.

The significance of the examination of bore holes with polarized light is difficult to evaluate. If it can be assumed (1) that hyphae penetrating by the application of mechanical force could cause minute failures in the penetrated cell wall, and (2) that such failures would be of sufficient size to produce interference patterns under polarized light, then the fact that they were absent in every bore hole examined would be proof that mechanical force is not involved. However, it is felt that any deductions

based on these assumptions would rest on uncertain ground. In the absence, then, of irrefutable evidence that mechanical force is not a factor, it seems safer to accept as conclusive the overwhelming positive evidence in favor of chemical penetration; at least until further facts are disclosed.

The Photographic Evidence

Reference is again made to Plates II to IX (inclusive) to emphasize the evidence upon which the above conclusion has been based. Plate II, showing the notch formed in the early stages of penetration, has been described. The typical constriction that usually accompanies penetration, and the relative size of the bore hole, are clearly shown in Plate III, in which the tip of the hypha has just reached the middle lamella. In Plate IV, the clearly defined cavity in front of the advancing tip offers strong support for the chemical theory. That the middle lamella is more resistant to the enzymes in this particular case, is evidenced by the reduced diameter of the bore hole at this point. Plates V, VI, and VII show the progress of hyphae after passing the middle lamella. The inevitable area of advanced corrosion and the relative size of the hyphae and bore holes are evident. The turn executed by the hypha in Plate VII is striking confirmation of chemical penetration. Plate VIII shows a hypha just in the act of emergence, with the bore hole visible along its entire length. The tip exhibits signs of enlargement; the first step in the resumption of normal size. Plate IX is one of the most striking pictures secured during the investigation. The hypha, which is magnified 2000 times, is only 1–1½ microns in diameter in the bore hole, yet the fact that the bore hole is considerably larger than the hypha is obvious. Plate X is included to call attention to the great care required in interpreting observations of penetrating hyphae. Actually, the end of the hypha in this picture is not seen; the form shown being the result of an oblique section of the hypha and bore hole. This occurs when the section is not cut in the longitudinal plane of the bore hole, or when only part of a curved hypha is cut. Such illusions are common, but all of the penetrating hyphae herein presented as evidence were examined with extreme care to insure that the end was clearly visible.

Plate IX may require additional explanation. It will be noted that the protruding hyphal tip is surrounded, or bounded, by a narrow whitish halo. The same phenomenon is present, though less obvious, in several of the other plates. This can arise from four causes: (1) halation, although

halations do not usually register in high-magnification photomicrographs; (2) diffraction; (3) the Becke line effect, which results from differences in the refractive index of an object and that of the medium in which it is suspended; and (4) by the presence of a gelatinous sheath around the hypha.

Diffraction is a distinct possibility, since it is virtually impossible to have all parts of a somewhat sinuous object such as a hypha in focus at the same point in the extremely shallow field that is obtained at such high magnifications.

The possibility of the presence of a gelatinous sheath cannot be dismissed. Even if it could be demonstrated that such sheaths do exist, however, the theory of complete chemical penetration would not be invalidated. It would not seem tenable to assume that such a sheath could be effective in transmitting a significant amount of mechanical force. It is possible that sheaths of this nature might occupy the free space in the bore holes and act in the transmission of enzymes. Yet even this is doubtful, since it does not seem likely that the activity of the enzymes could be restricted to the outer surface of such a sheath.

The Becke line effect is used by petrographers to determine the index of refraction of an unknown material. This is accomplished by suspending a small particle of the material in a medium of known index and observing the position of the Becke line when the objective of the microscope is raised or lowered. If the bright line moves from the medium to the object when the objective is lowered, the index of the object is lower than that of the medium, and vice versa. By mounting in several known media, the index of the object can be determined. Thus, it can be seen, that such an effect could be obtained if the indexes of the hyphal wall and the mounting medium were in the proper relation to each other, or if they were in the exact focus that would produce the Becke line.

It is impossible to determine with positive assurance which of these phenomena may have produced the described effect but it can be affirmed with considerable confidence that neither one alone, nor all acting concurrently, could have any direct bearing on the conclusions that have been drawn.

The original presentation of the dissertation on which this paper is based contains many additional photomicrographs having either direct or indirect bearing on the subject of penetration. Limitations of space have precluded their inclusion in full in this publication, but fourteen supplementary plates have been added to permit a more complete devel-

opment of the evidence disclosed by the investigation. Reference has been made to these plates during the course of the text. In order to avoid duplication of explanatory material each plate has been provided with an individual description which has been designed to call attention to the important features.

In presenting the evidence disclosed by this investigation, it is desired to emphasize that there has been no intention of minimizing the excellent work done by Cartwright. While the evidence for total chemical penetration has been strongly fortified and the case for mechanical penetration materially weakened, it must be remembered that the latter theory has not been conclusively disproven. In the light of the wide scope of his investigations, the materials with which he worked, and the methods and equipment at his disposal, his hypothesis was by no means unreasonable.

Identification of Fungi by Their Hyphal Characteristics

Examination of the large amount of material made available by this investigation afforded an excellent opportunity to evaluate the possibility of identifying fungi by their hyphal characteristics in decayed wood. A study of over five hundred slides and two hundred and fifty photomicrographs of hyphal features has indicated an unqualified assertion that such a method would be entirely feasible. That this would be desirable has been voiced by many pathologists.

Present methods of identification, in the absence of sporophores on the decayed wood, depend on cultural and macroscopic characteristics. The former is often extremely slow and requires the maintenance of a large collection of stock cultures for comparison. Gross characters of decay may be highly variable.

Further studies to establish diagnostic criteria would be very desirable. The original work of Hubert (1924), and the thorough compilation by Bavendamm (1936) would provide a starting point.

SUMMARY

EARLY investigators in the field of forest pathology recognized and recorded the passage of fungal hyphae through the walls of wood cells; and the fact that this was accomplished, for the most part, by enzymes secreted by the fungus was early recognized. The actual facts dealing with the mechanisms of such penetration had, however, never been established and recent statements suggesting the possibility that

mechanical force might be a contributing instrumentality of penetration prompted a thorough investigation of the subject.

Analysis of the methods employed by fungi in the penetration of plant tissues disclosed that two may be used by the hyphae of xylophagous fungi in penetrating the thick, lignified walls of wood cells. These are: (1) penetration by a complete, local dissolution of the cell wall by the action of enzymes secreted in advance of the penetrating hypha, and (2) penetration by the application of mechanical force after enzymic activity has effected a general softening of the wood in the vicinity of the penetrating hypha. The present investigation was undertaken in an attempt to establish the facts of hyphal penetration by wood-destroying fungi, and to determine, if possible, the relative importance of the two methods by which it has been assumed that such penetration might be accomplished.

The steps in the organization of the problem follow:

1. The preparation of decayed wood by the inoculation of culture blocks with a suitable number of representative fungi.

2. The sectioning of the wood blocks and the preparation of microscope slides for purposes of observation.

3. A careful search of all material for any facts pertaining to the processes of penetration.

4. Obtaining an irrefutable record of observations in the form of photomicrographs.

Decayed wood for the investigation was obtained by inoculating one hundred and thirty-eight blocks, representing four species of coniferous woods with pure cultures of six representative species of wood-destroying fungi.

The study of fungi in decayed wood presents very difficult technical problems. Fungal hyphae are microscopic in size and often colorless. This necessitates differential staining and the difficulty of staining fungi in wood is patent to all who have attempted it. Sharp counter staining is a requisite of successful photomicrography with visible light, and since this is impossible, satisfactory photographic evidence is difficult to obtain. Further, nearly all usual methods of preparing sections for microscopical examinations require dehydration of the material. This results in shrinkage, distortion, and hardening, any one of which is prejudicial to the preservation of natural conditions. The same objections are inherent to the usual methods of embedding.

Not the least of the problems encountered was the finding of hyphae in the actual act of penetration, a detail which has not often been observed,

and never before photographed, at least, insofar as a careful search of available publications has disclosed.

The importance of these obstacles can be appreciated when it is realized that the success of the entire investigation depended on the accurate natural reproduction of microscopic detail. These difficulties were overcome to a large extent by a technique developed and perfected in this, and a preliminary investigation. The essential features contributing to success were:

1. The use of ultra violet photomicrography with the 3650 A° spectral line to record all observations. This relatively new method provides increased resolution of the microscope. Higher magnifications, without loss of detail, are made possible and only sufficient staining to permit the location of desired details is required.

2. The use of histological methods designed to preserve natural conditions to the highest possible degree. Water soluble stains and a water soluble mounting medium were used to avoid dehydration and its undesirable consequences. Embedding of the material was avoided in every possible case, since customary methods of infiltration embody the same undesirable features which have been attributed to dehydration.

Through the use of this technique, more than five hundred microscope mounts were prepared and examined, and approximately two hundred and fifty photomicrographs of individual features were obtained from one hundred of the mounted sections. A series of eight carefully selected photomicrographs showing the stages of the penetration of a cell wall by the hyphae of wood-destroying fungi is presented as evidence in support of the theory of total chemical penetration. Fourteen additional photographs have been included to emphasize important steps in the evidence.

CONCLUSIONS

FROM the evidence obtained, it is concluded that the penetration of the walls of wood cells by the hyphae of wood-destroying fungi is accomplished by (1) the secretion of enzymes at the tips of penetrating hyphae and (2) the total, local dissolution of the cell wall by enzymic activity in advance of actual passage through the cell wall. Thus, penetration is effected through a preformed passage without actual contact between the hypha and the penetrated cell wall, with the possible exception that contact with the cell wall at the very first point of penetration may be the stimulus which initiates enzymic activity. In all cases of penetration ob-

served, the tip of the hypha was preceded by a cavity of significant proportions. This is clearly shown in the photomicrographs.

Careful examination of hundreds of bore holes, some with polarized light, failed to disclose evidence of any kind that mechanical force is an instrumentality in the penetration of the cell walls of wood, although it must be admitted that the evidence is of a negative nature.

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ILLUSTRATIONS

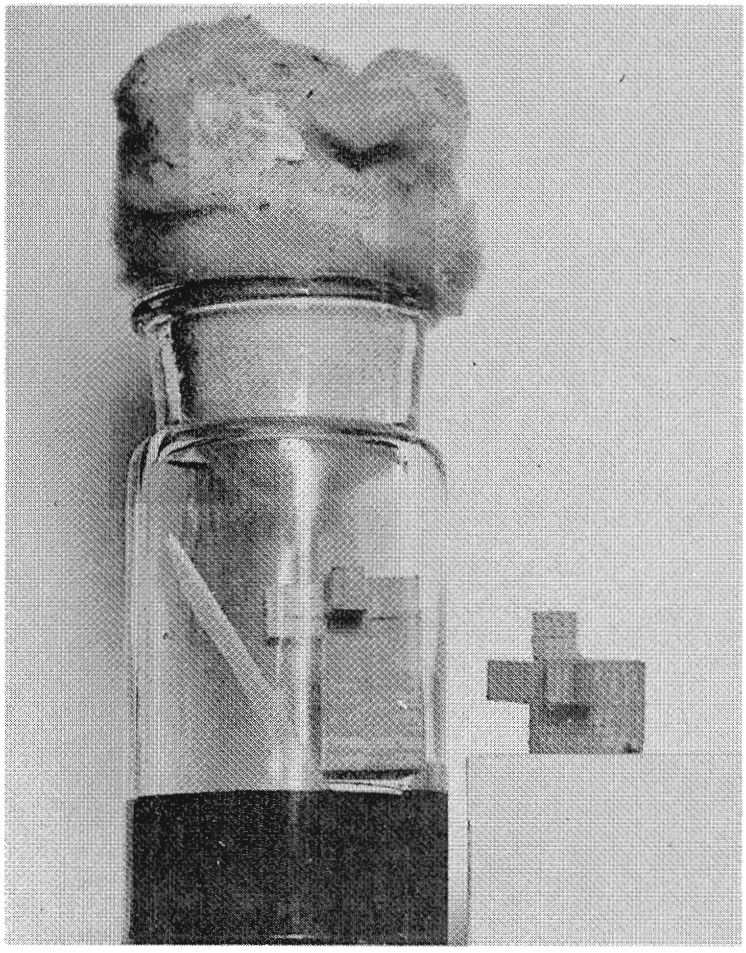

PLATE I.

Method of placing wood blocks in culture bottles. The microscope slide prevents the block from coming into contact with the agar while the culture is being sterilized. A pre-cut block is seen at the right. The advantages of this type of block are described in the text. × ¾.

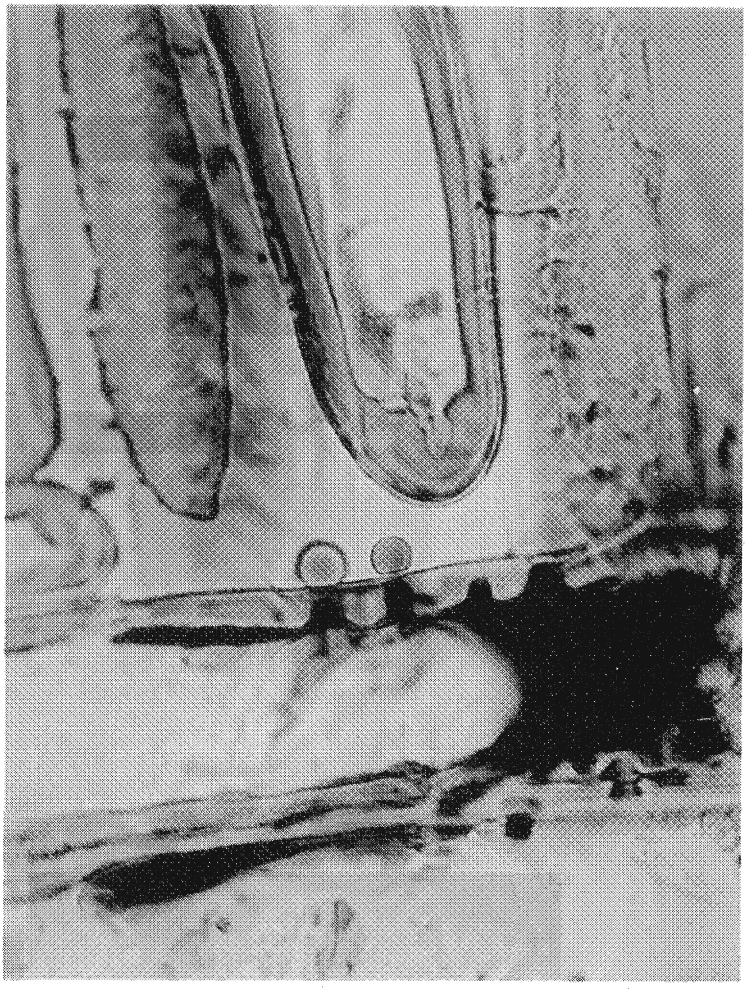

PLATE II.

Lenzites trabea in western hemlock. An early stage of penetration. The constricted point of a relatively large hypha can be seen occupying a notch formed in the thick secondary wall at the end of a tracheid. Chemical corrosion is evident in the cell wall at the right. × 1640.

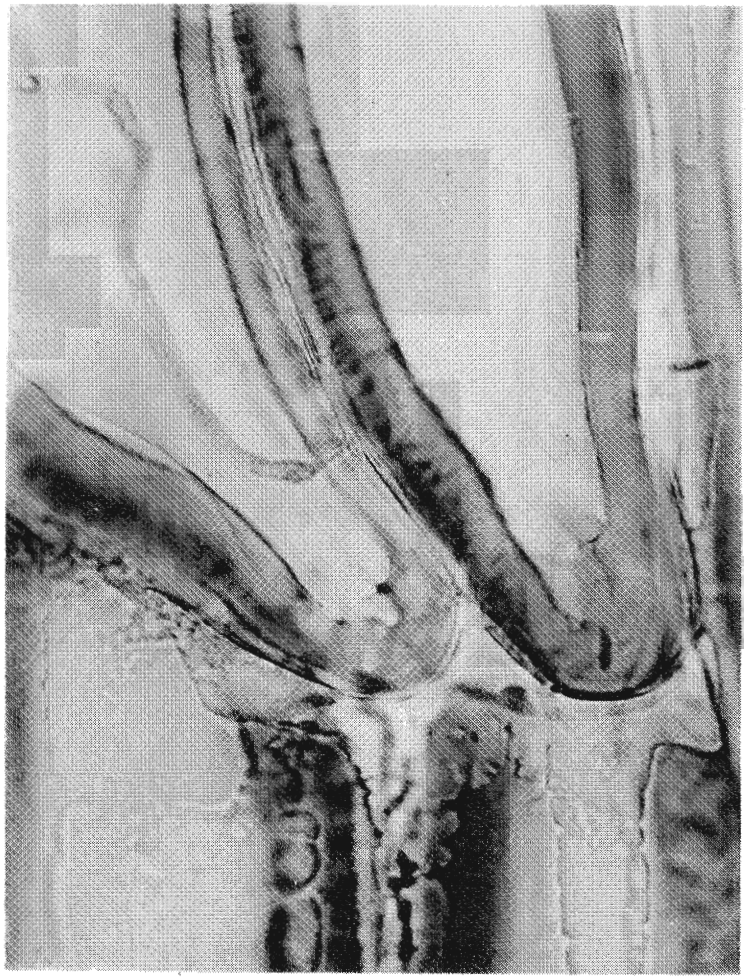

PLATE III.

Lenzites trabea in western hemlock. The typical constriction that accompanies penetration by larger hyphae. The advancing tip has just reached the middle lamella. The bore hole is plainly visible. × 1620.

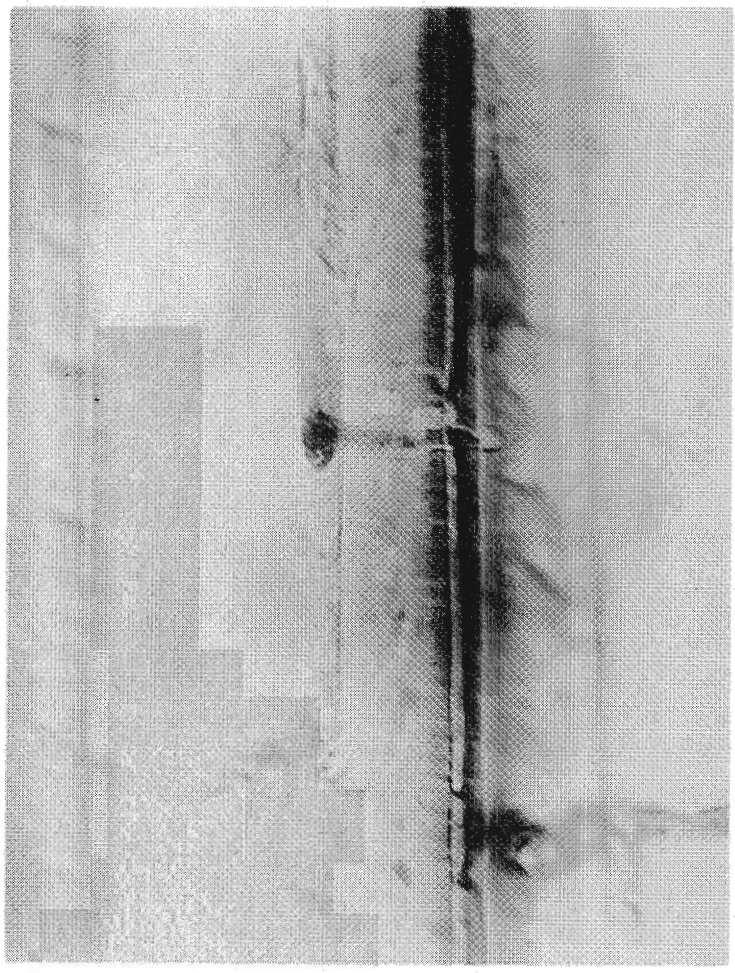

PLATE IV.

Trametes serialis in western hemlock. A hypha just passing through the middle lamella. Protoplasm is concentrated in the tip of the hypha and there is a cavity immediately in front of the tip. The fact that the middle lamella, in this particular instance, is more resistant than the secondary wall is evidenced by the reduced diameter of the bore hole as it passes through the middle lamella. × 2220.

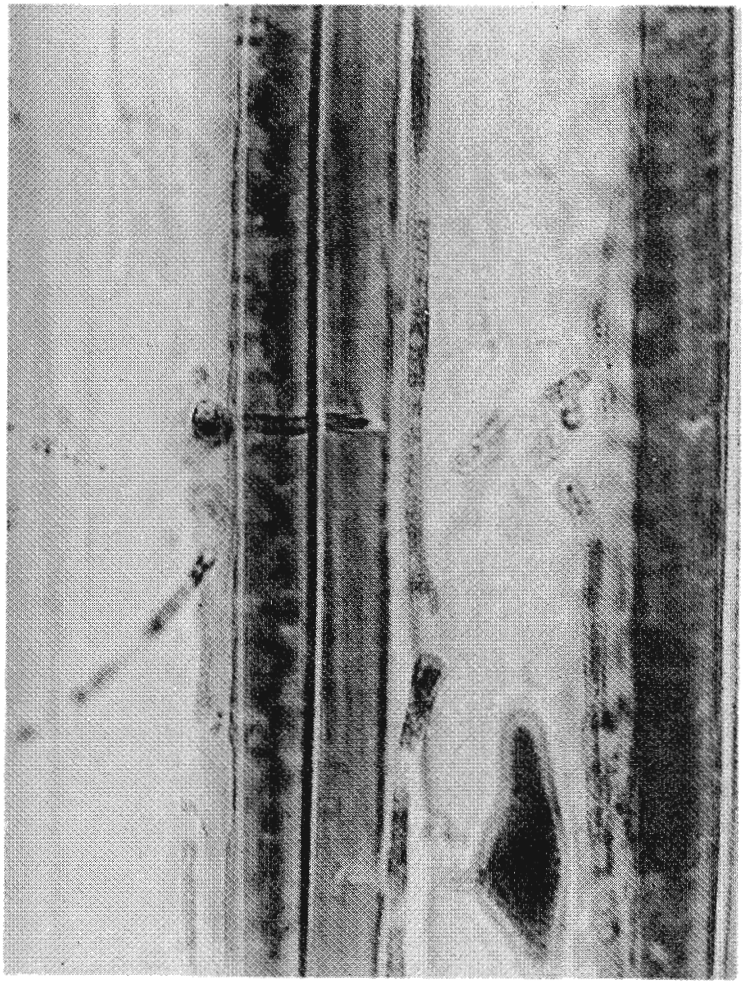

PLATE V.

Trametes serialis in western hemlock. A hypha that has passed the middle lamella. The cavity in advance of the hypha is distinct and the bore hole, while not clear, is visible. × 1680.

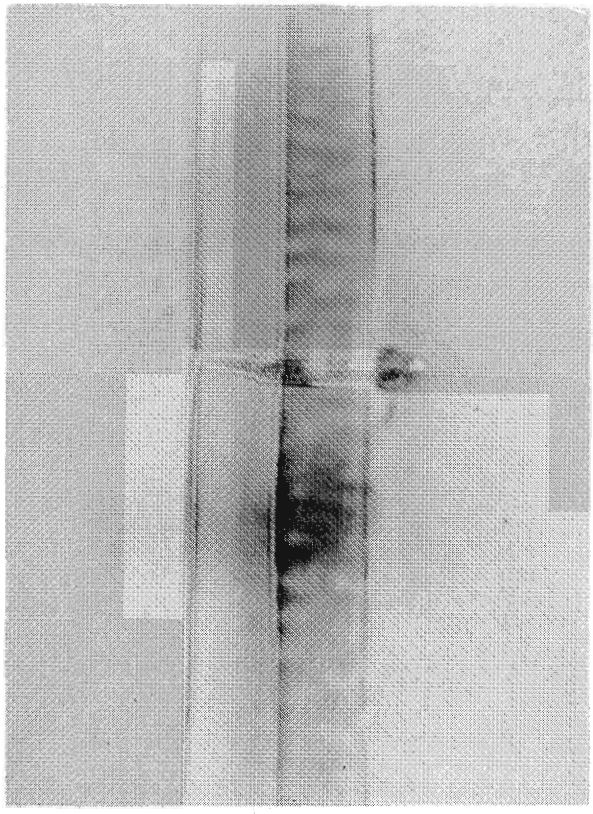

PLATE VI.

Trametes serialis in western hemlock. A hypha approaching the point of emergence. The relative size of hypha and bore hole is clearly shown, as is the well-formed tunnel in advance of the tip. × 2000.

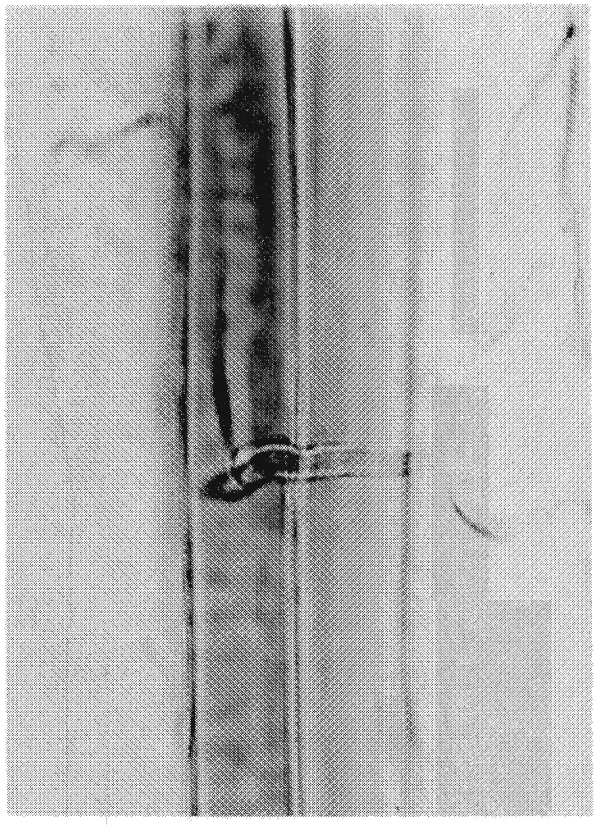

PLATE VII.

Trametes serialis in western hemlock. The turn made by this hypha is striking evidence in support of the chemical theory of penetration. The enlargement of the bore hole at the turn is also indicative of chemical action. × 2000.

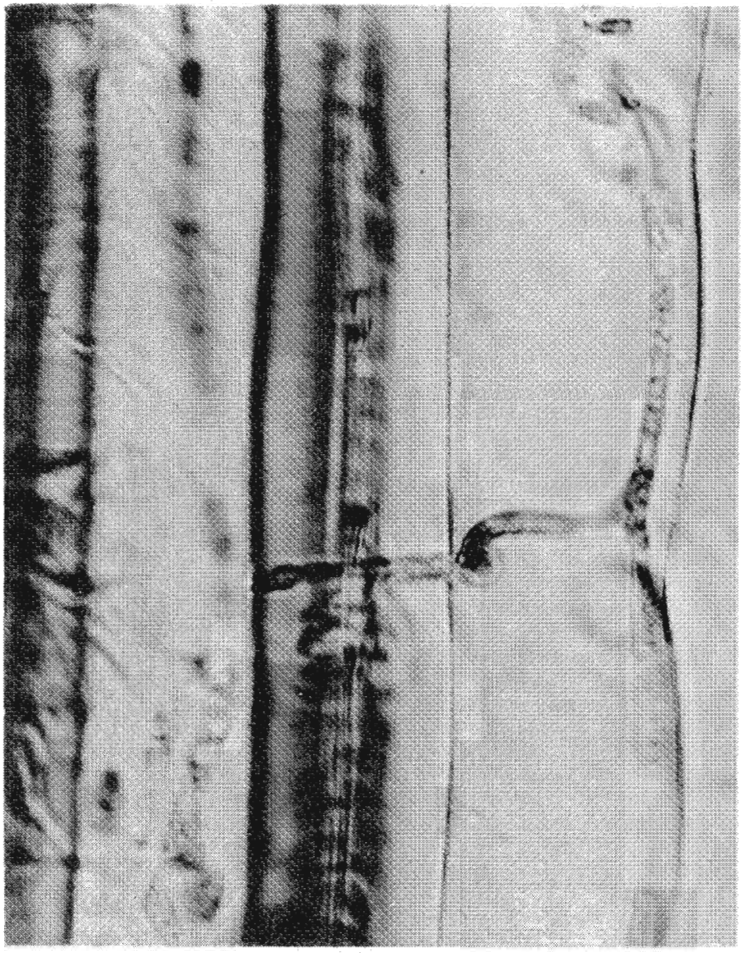

PLATE VIII.

Trametes serialis in western hemlock. A hypha in the act of emergence. The tip appears to have begun to enlarge before actual passage has been accomplished. (See text for a discussion of the white halo around the tip of the hypha.) × 1610.

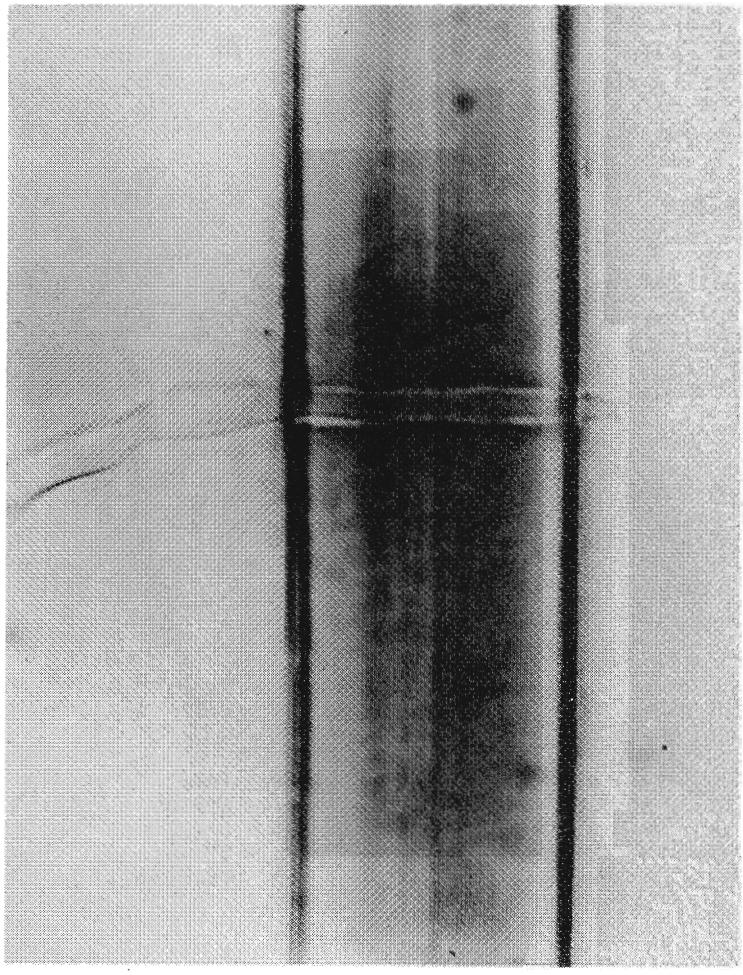

PLATE IX.

Trametes serialis in western hemlock. A small hypha that has just completed the penetration of a cell wall. The hypha is 1–1.5 microns in diameter in the bore hole. Only a very high magnification would disclose the presence of the bore hole. (See text for a discussion of the possible cause of the halo around the protruding tip.) × 2240.

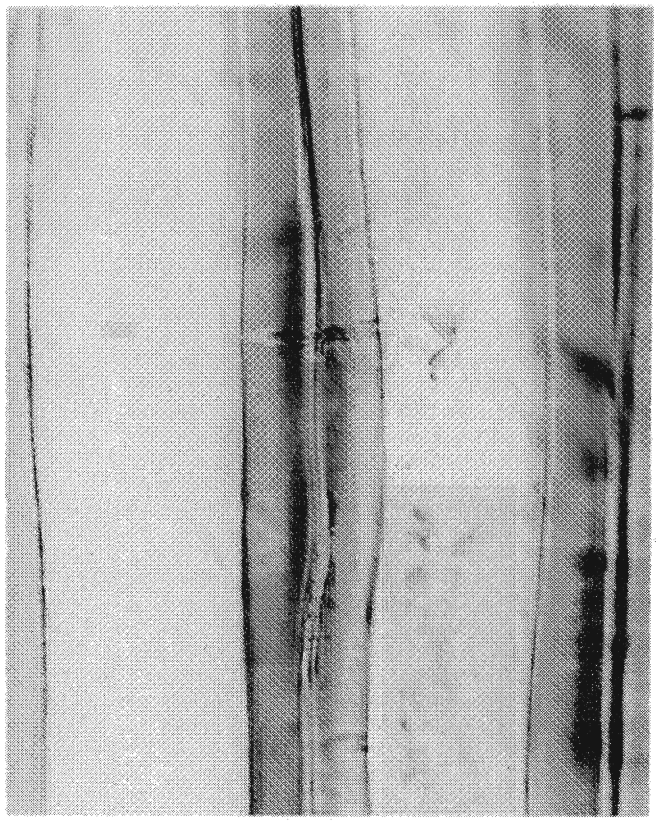

PLATE X.

Trametes serialis in western hemlock. A special photograph included to call attention to a possible source of error in observations of penetrating hyphae. The end of the hypha shown is not visible in the picture. The illusion is formed as the result of an oblique section through both the hypha and the bore hole. This occurs whenever the section is cut at an angle with the longitudinal axis of the bore hole or when only a part of a curved hypha is cut. All of the hyphae submitted in evidence were carefully examined to be sure that the extreme tip was clearly visible. × 1500.

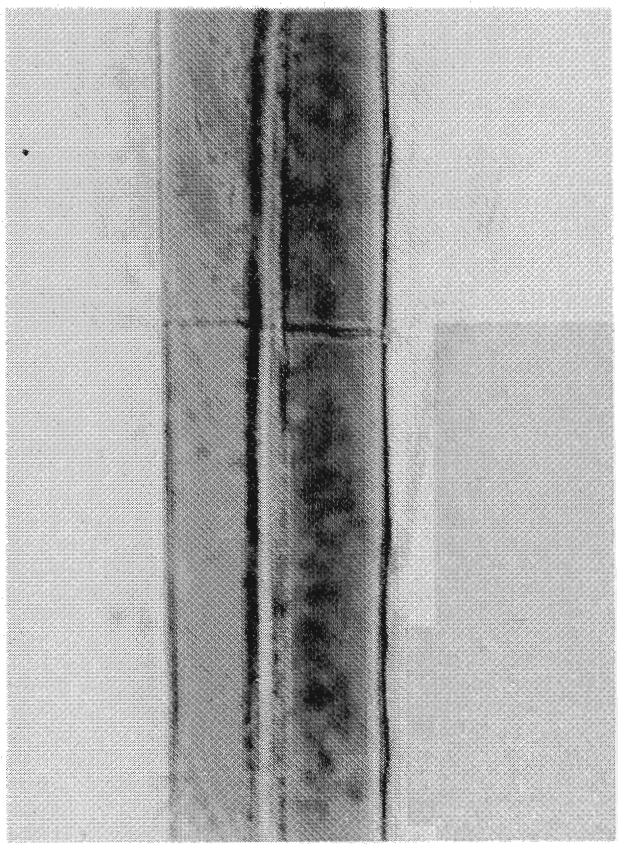

PLATE XI.

Fomes pini in western hemlock. Completed penetration by a small hypha. The hypha is approximately 0.5 micron in diameter in the bore hole, yet the bore hole is visibly larger than the hypha. Marked constriction occurs at both the point of entrance and the point of exit. × 2000.

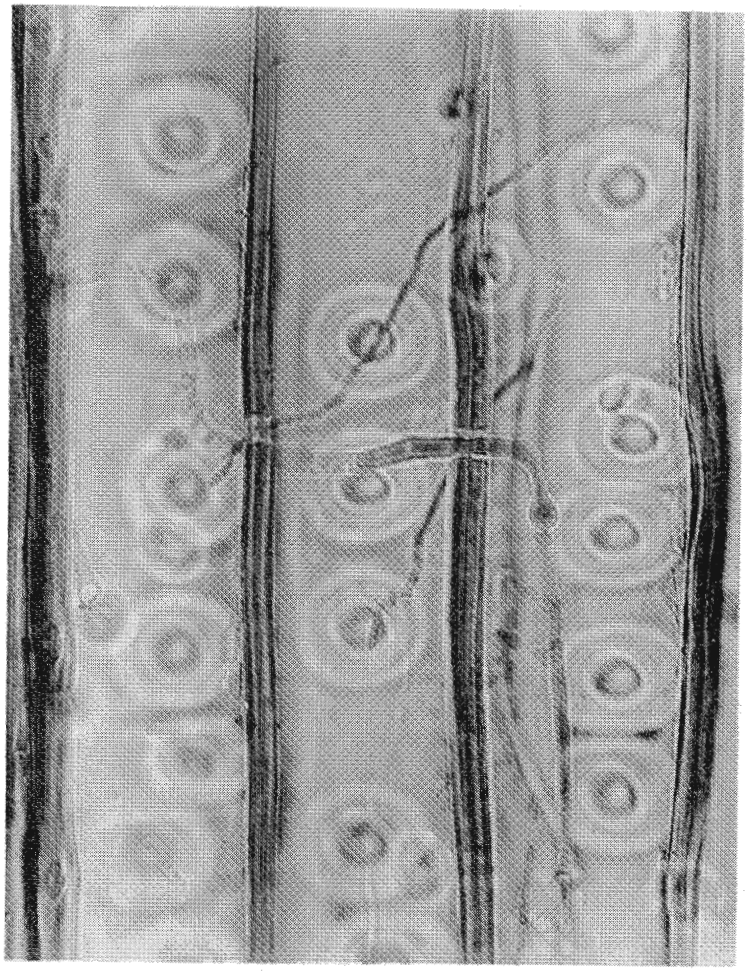

PLATE XII.

Fomes pini in eastern white pine. Completed penetration by a large hypha. The small hypha seems to occupy the bore hole from which the large hypha has been displaced, although it is likely that it was dragged into this position during sectioning. × 830.

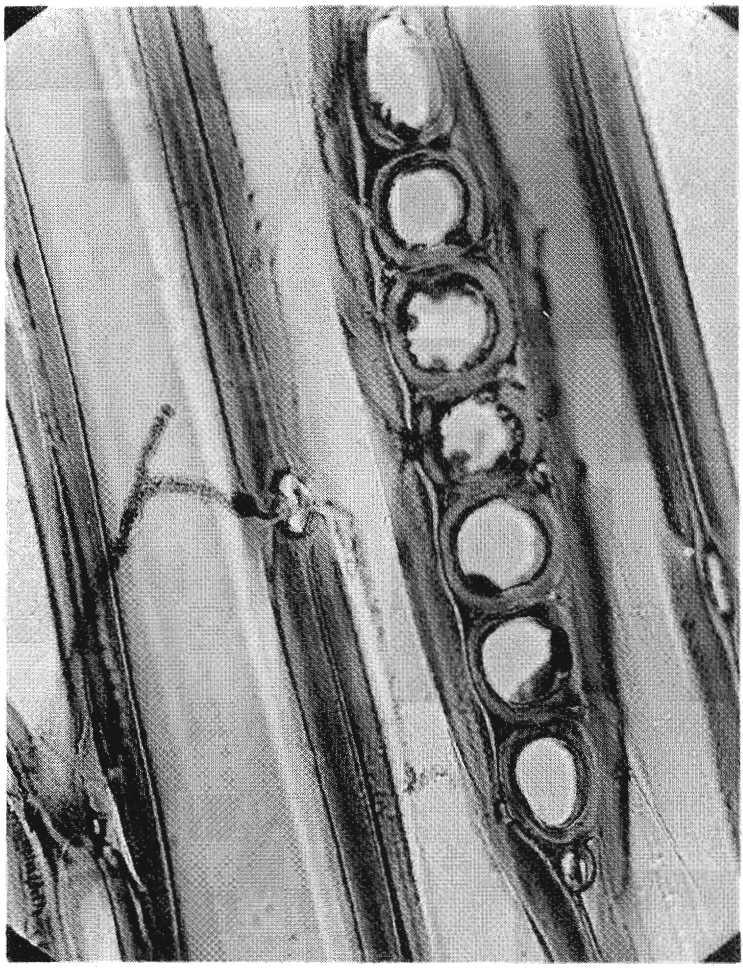

PLATE XIII.

Lenzites trabea in western hemlock. Penetration of a bordered pit-pair as seen in tangential section. Protoplasm is concentrated in the sharply constricted point where the hypha enters the pit. This indicates that the use of pits as avenues of transmigration does not involve a special means of penetration (see Plate XIV). Most of the hyphae seen penetrating pits avoid the torus in favor of the pit membrane. × 830.

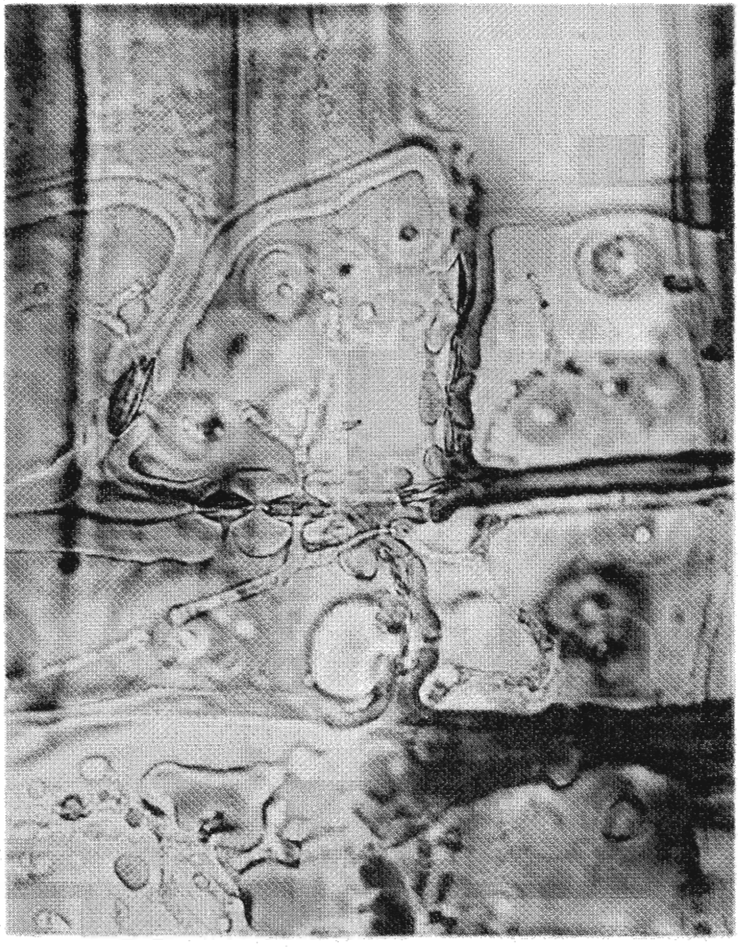

PLATE XIV.

Fomes annosus in eastern white pine. The hypha traversing the horizontal wall between two ray tracheids (center) has passed directly through a bordered pit-pair. The hypha just to the right of this has passed directly through the cell wall only a short distance from a pit-pair. The hypha in the vertical wall (upper left) has "bored" through the border on one side and completed the penetration through the chamber and aperture of the pit on the opposite side (see Plate XIII). × 1610.

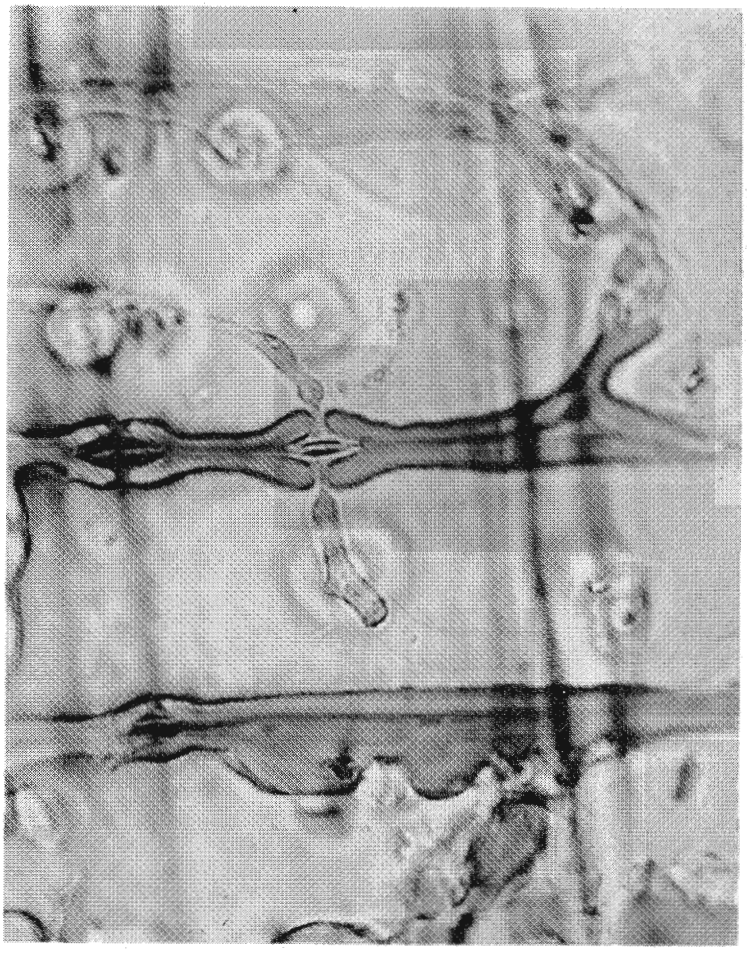

PLATE XV.

Fomes annosus in eastern white pine. This highly magnified hypha clearly shows the typical constriction of penetrating hyphae. Since no bore hole is visible in the torus, it is likely that the passage was effected through the pit membrane. While not focused to bring out details of the pit-pair, it clearly shows the duplex structure of what is often considered a single torus (in reality, paired tori of complementary pits). × 2160.

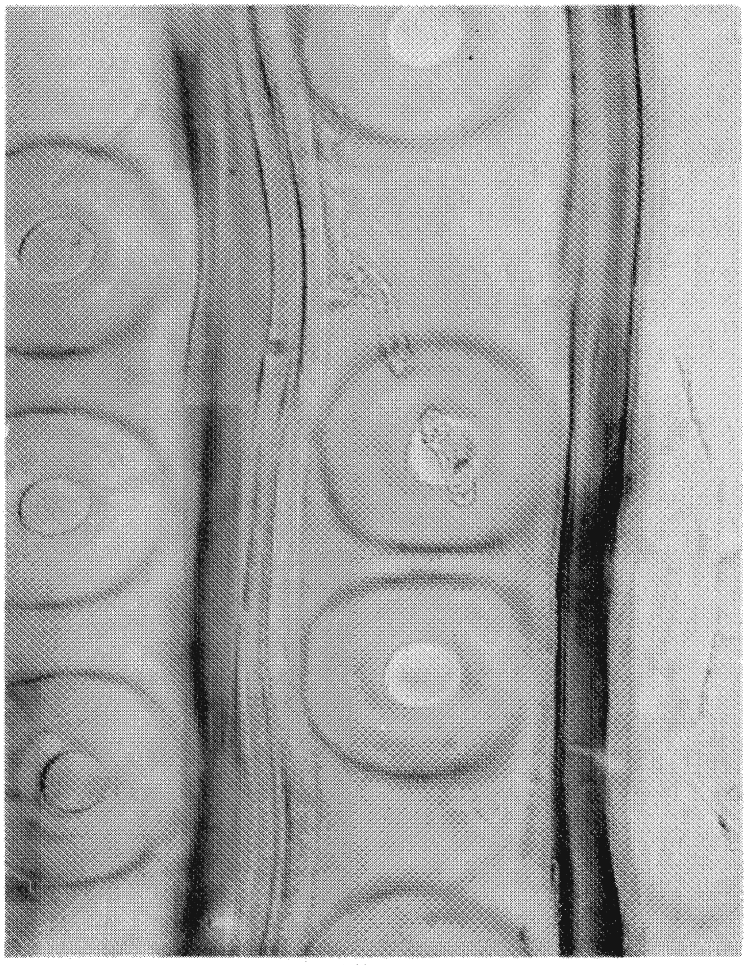

PLATE XVI.

Fomes annosus in eastern white pine. This picture shows a hypha penetrating only the border part of a pit. This is similar to the condition described in Plate XIV. × 1660.

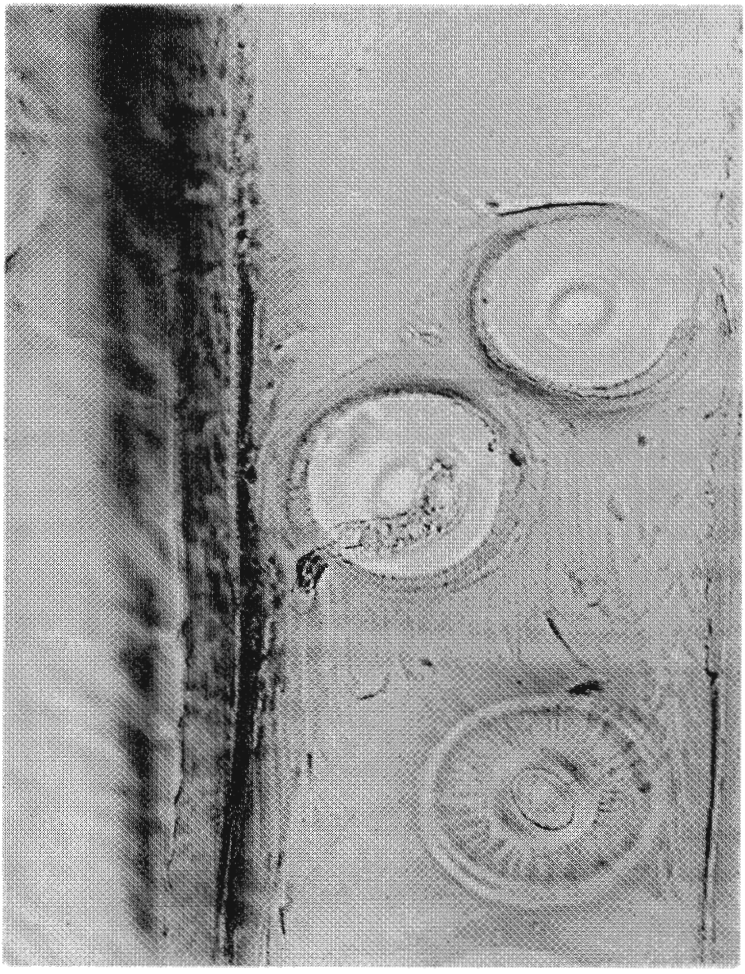

PLATE XVII.

Trametes serialis in Douglas fir. A hypha emerging from a bordered pit and doubling back through the cell wall. The border of the pit has been completely dissolved by enzymatic activity. In the lowest pit the structure of the membrane is shown more clearly as a result of the chemical action of the fungal enzymes. × 1650.

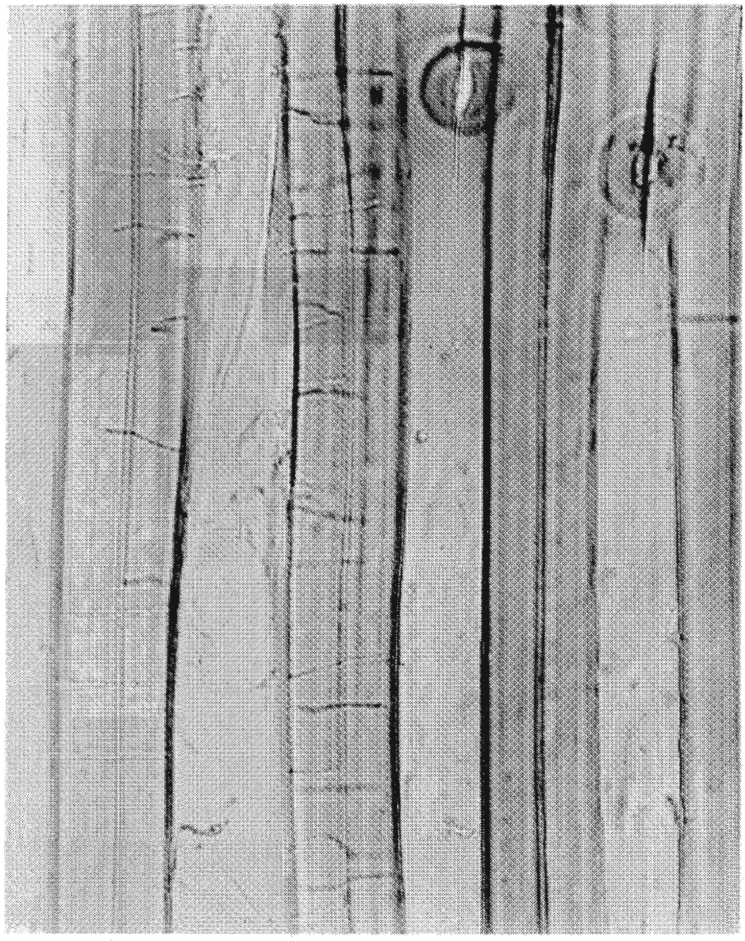

PLATE XVIII.

Fomes annosus in eastern white pine. Typical bore holes produced by the small, actively "boring" hyphae of *Fomes annosus*. The sinuous form suggests chemical penetration. Extended apertures of bordered pits can be seen in the upper right-hand corner. × 1070.

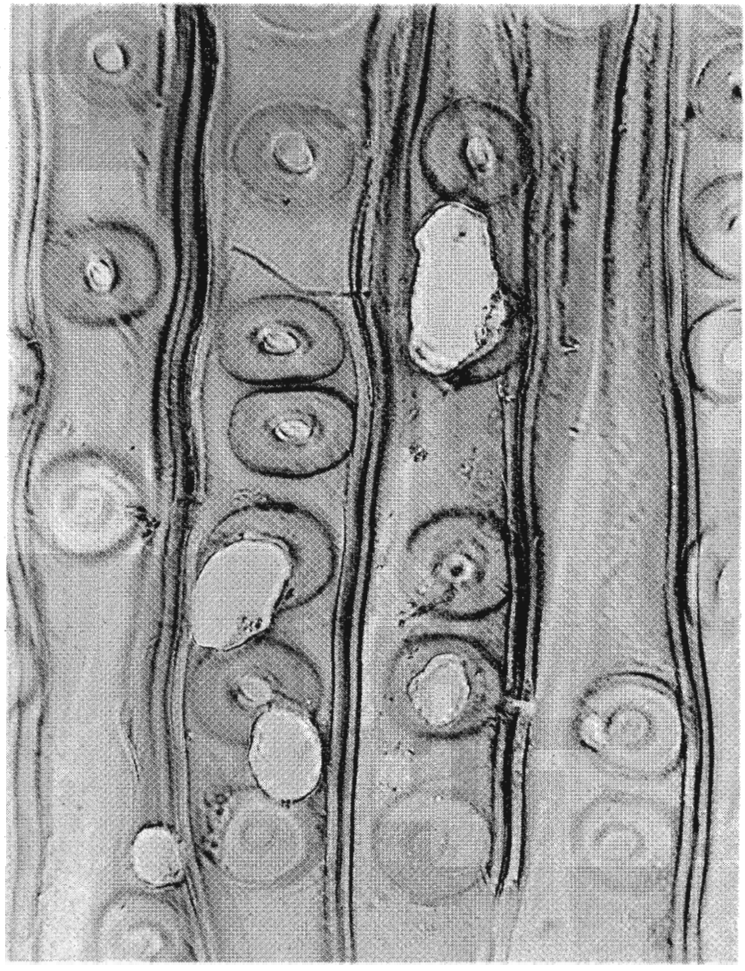

PLATE XIX.

Fomes annosus in eastern white pine. General dissolution of bordered pits and cell walls by enzymes. While this condition may result from the diffusion of enzymes through existing bore holes, it cannot be considered as the enlargement of bore holes in the sense that the latter expression is usually used. × 820.

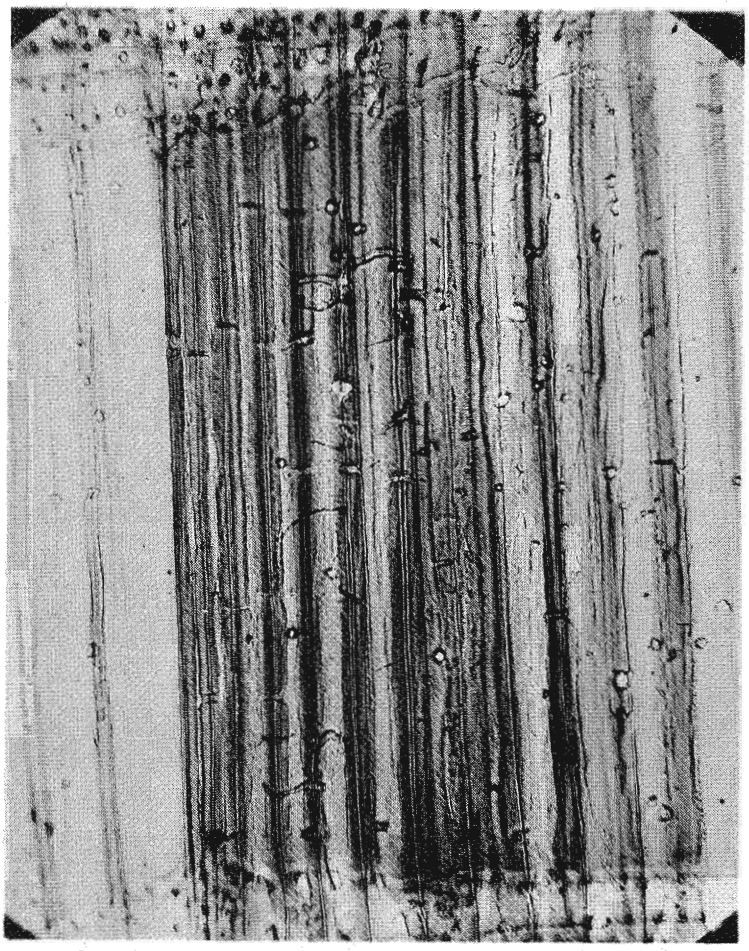

PLATE XX.

Fomes pini in eastern white pine. A general low-power view of wood in the first stages of advanced decay. Hyphae are absent, a common condition in the later stages of decay. Bore holes of various sizes, all with smooth, corroded sides, give evidence of the part played by enzymes in their formation. The sinuous bore holes serve particularly to emphasize this point. × 380 (dry objective).

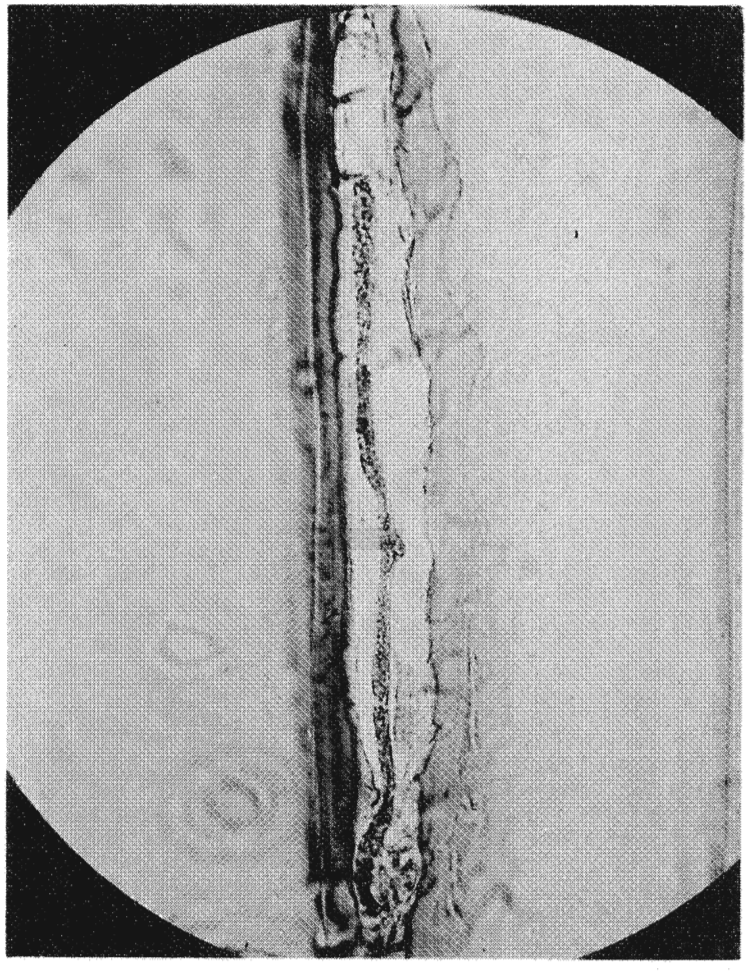

PLATE XXI.

Trametes serialis in western hemlock. A hypha "boring" directly along a middle lamella. While *Trametes serialis* is a brown rot (cellulose-consuming) fungus, the enzymes secreted from the tips of the hyphae seem to dissolve lignin without difficulty. × 1090.

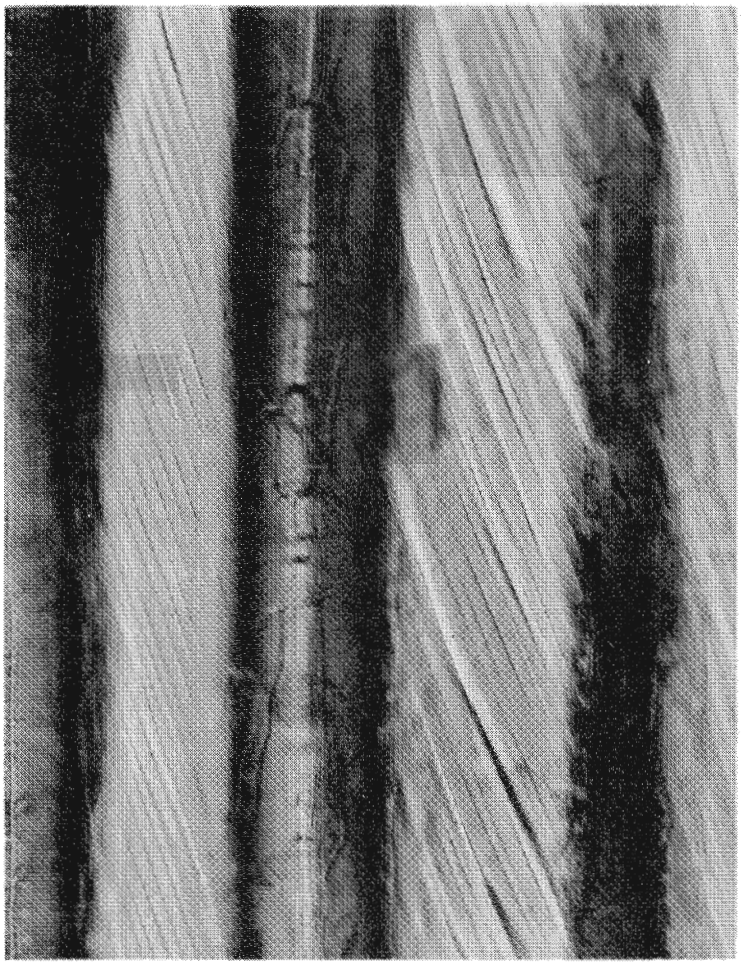

PLATE XXII.

Fomes pini in Douglas fir. The spiral cracks, which result from a general weakening of wood by decay, are believed to assume the angle of the fibrils of the middle layer of the secondary wall. × 1100.

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