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Development and Composition of the Warty Layer in Balsam Fir [*Abies balsamea* (L.) Mill.]

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DEVELOPMENT AND COMPOSITION OF THE WARTY LAYER IN BALSAM FIR [*ABIES BALSAMEA* (L.) MILL.]

A thesis submitted by

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SUMMARY

The warty layer is an amorphous structure containing numerous protuberances and covering the inner cell wall surface of the longitudinal tracheids of most softwoods and the vessel elements and sometimes the fibers of a few hardwood species. Electron microscopy and various analytical chemistry techniques were employed to study the development and composition of the warty layer in a softwood, balsam fir [Abies balsamea (L.) Mill.].

Samples of living tissue were taken during a period of active growth, chemically fixed to preserve cell constituents, then sectioned and stained for electron microscope observation. The warty layer was observed to be gradually developed external to the plasma membrane after secondary wall deposition and the greater part of lignification were complete. Warts developed first in cell corners and pit cavities and then on the radial and tangential cell walls nearly simultaneously. No organelle within the cytoplasm was found to be associated with wart formation. After the warty layer was elaborated, the cytoplasm disappeared from the cell without leaving any apparent, disorganized residue. The bulk of the wart structure had similar staining characteristics to lignin; however, the basal portions of individual warts were sometimes less darkly stained than the outer portions.

Examination of the inner surfaces of developing tracheid walls confirmed the observations recorded from the parallel study of wall sections. In the course of these examinations, it was also found that the permeable margo region in the membrane of mature bordered pits arose from a gradual perforation of an initially solid membrane. The perforation process occurred at the end of cell differentiation after wart formation was complete and was likely associated with cell autolysis.

The composition of the warty layer was determined by using the electron microscope to monitor the results of various chemical, physical, fungal, and enzymatic treatments on mature balsam fir wood. From the response of the warty layer to these treatments, it was concluded that the warty layer consisted largely of a ligninlike material. The interior, basal component of individual warts and some of the accompanying encrustant on the inner surface of the secondary wall consisted of an amorphous carbohydrate, probably a pentosan or pectic substance. While the bulk of the warty layer was definitely ligninlike in chemical reactivity, it was more resistant than a significant portion of the other lignin in the cell wall, even though the warts were the cell wall component most accessible to the treatment solutions. From the results, it appeared that the lignin in the warty layer was more concentrated and more condensed than lignin in other parts of the cell wall. Vacuum drying at 105°C condensed the wart structure even further, making it even more resistant to most treatments.

Reagent solutions found to dissolve the warty layer were analyzed in an attempt to determine the specific composition of this structure. The warty layer in solution had UV absorbance similar to lignin, or, if it was different, any such indication was masked by the extracted lignin from other portions of the cell wall also in solution. Different chromatographic analyses of extraction solutions failed to reveal any unique components associated exclusively with the warty layer. The analyses did indicate that the warty layer was extracted as a high molecular weight material by at least some treatments. Attempts to physically isolate the warty layer were largely unsuccessful.

Three alternative hypotheses are offered to explain the development and composition of the warty layer in balsam fir:

1. Warts are vestiges of sites of material transport from the cytoplasm to the developing cell wall.

2. Warts are formed by deposition through the plasmalemma of autolysis products of the dying cell with no relation to sites of previous deposition of cell wall components.

3. While less likely, the wart structure may be due to an eruption of material from the cell wall into the lumen caused by localized areas of high osmotic potential.

Several additional observations were made to fulfill secondary objectives of this research. Evidence was obtained to indicate that the chemically resistant warty layer may act as a barrier to the penetration of liquids into the cell wall, thereby causing different delignification rates among species depending on the nature of the inner cell wall surface. In cells of hardwood species, the presence or absence of a warty layer may follow a trend that can be generally associated with the degree of phylogenetic advancement of the cell type. Within hardwoods containing both vessel elements with scalariform perforation plates (more primitive) and simple perforation plates (more advanced), warts, when present, were most often associated with the former vessel type.

INTRODUCTION

The wood cell wall is the fundamental natural resource of the pulp and paper industry. As such, an understanding of its structure and composition is important even at the most minute levels to facilitate the intelligent and efficient use of this raw material. This thesis research was undertaken to explain the origin, nature, and significance of one feature of the wood cell wall — the warty layer which lines the interior (lumen) surface of the cell wall.

HISTORICAL REVIEW

In many wood cells the surface of the wall adjacent to the lumen consists of a bumpy covering known as the warty layer. Figure 1 is a cutaway model of a mature softwood tracheid showing the appearance and location of the wart structure (W). The existence of this structure was first reported in 1951 by Kobayashi and Utsumi (1) and by Liese (2). It is one of the very few basic cell wall components that was discovered only after the application of the electron microscope to the study of wood ultrastructure.

Experimental observations and theories on the structure, occurrence, composition, origin, and significance of the warty layer have been reviewed by Frey-Wyssling in 1957 (3), Liese in 1963 (4), Wardrop in 1964 (5), Liese again in 1965 (6) and Katkevich and Milyutina in 1972 (7). Of these, the 1965 review by Liese is the most comprehensive. The review by Katkevich and Milyutina in Russian has the advantage of presenting the most recent literature. Some experimental observations on the warty layer, most notably those of Scurfield, et al. (9-14), have been published very recently and are not included in any review.

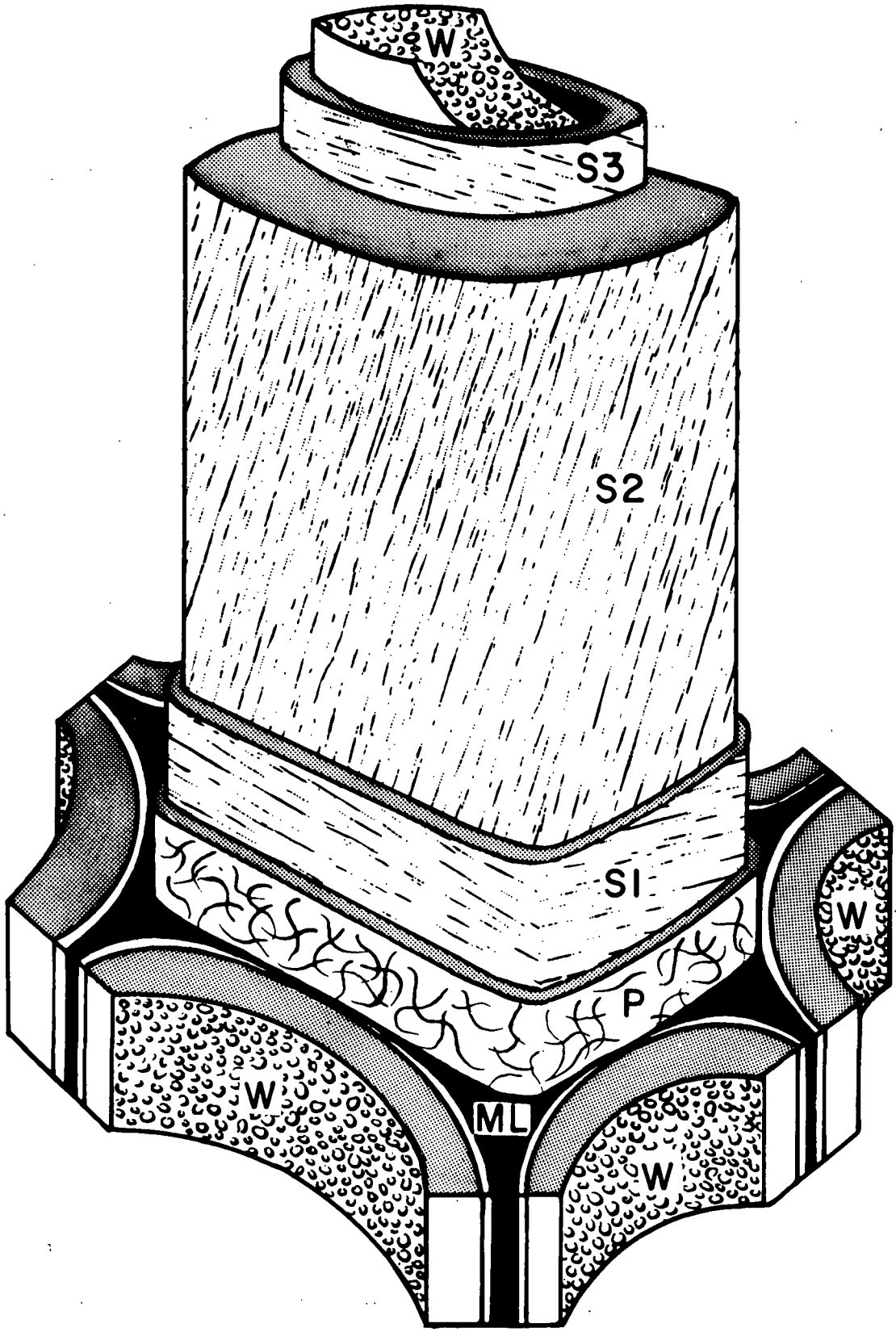


Figure 1. Cutaway Model of a Mature Softwood Tracheid Illustrating Cell Wall Layers: ML = True Middle Lamella (Intercellular Substance); P = Primary Wall; S1, S2, and S3 = Outer, Middle, and Innermost Layer of the Secondary Wall, Respectively; W = Warty Layer. From Côté (8)

STRUCTURE

The warty layer is an amorphous layer of material lining the inner surface of the secondary wall in cells of many wood species. This layer contains numerous protuberances — hence the name, warty layer. In some species the warts appear to lie directly on the innermost portion of the secondary wall, S3, with very slight or no additional encrustant. A few other species possess an amorphous layer as a lining on the cell lumen and very few or no protruding warts. Usually, however, the warts and amorphous layer are found in combination.

The distribution of warts can be quite random in cells that exhibit them. Individual warts vary in shape from nearly flat mounds in some species to high, fingerlike projections in others. Wart diameter reportedly ranges from 0.01 μm to 1.0 μm (15). Within one species, however, the range is usually relatively narrow, and the average size lies between 0.1 μm and 0.25 μm (6). Other physical characteristics found for the warty layer are a refractive index of 1.52 and the ability to absorb ultraviolet light (16).

OCCURRENCE

The warty layer is found in the longitudinal tracheids of many softwoods and in the vessels and sometimes the fibers of a few hardwood species, lining the lumen and pit structure in these wood cell types. The occurrence of warts is reported not only for softwood and hardwood xylem, but also for the vascular cells of herbaceous plants (17) and softwood needles (4). The warty layer is not restricted to normally differentiated cells since it also appears in compression wood of softwoods (18, 19) and tension wood fibers of some hardwoods (20), although the evidence here is not as strong.

True wart structure apparently occurs only in lignified tissue, though there is some controversy on this point. The lumen of cotton seed hair, an unlignified cell, exhibits a granular structure on the cell wall surface consisting of disorganized cytoplasmic debris as shown by fluorescence and electron microscopy (21, 22). Azzola, et al. (22) indicated that this material is analogous to the warty layer. Their association is based on the supposedly common origin of these deposits in cotton and the warty layer in wood. This supposition is questionable, as will be shown later. Also, the debris in cotton bears little resemblance to the protuberances into the lumens of wood cells. Previous to Azzola's work, Rollins (23) stated clearly, "nothing resembling the warty layer has been identified in cotton fibers."

Warts appear in only some wood cell types, and they can be quite variable from species to species in size and distribution pattern. This raises the question of whether or not the warty layer has some taxonomic significance. Liese (6) has summarized observations on the appearance of the warty layer in a large number of gymnosperms. The data, along with a few contradictions by other workers, are presented in Table I.

Within most softwood genera, all species possess a similar trend for warty layer appearance (or absence). The genus Pinus, however, shows a distinction between the Diploxylon species (hard pines) which possess a distinct warty layer and the Haploxylon species (soft pines) in which warts are rare or absent (24-27). It is of interest to note that the presence of dentate ray tracheids in pine follows an identical pattern.

Ohtani and Fujikawa (28) studied the warty layer in sixteen softwood species and found that variability of wart number, size, and shape within a

TABLE I

OCCURRENCE OF A WARTY LAYER WITHIN THE GYMNOSPERMS (6)

Family	Genera	Species Examined	Appearance[a]
Cycadaceae	Cycas	1	0
Ginkgoceae	Ginkgo	1	0
Pinaceae	Abies	10	+
	Pseudotsuga	1	0
	Tsuga	5	+
	Picea	11	0
	Pseudolarix	1	0
	Larix	4	0(+,30)
	Cedrus	2	+
	Pinus:		
	Subgenus		
	Haploxylon	19	0
	Diploxylon	62	+
Taxodiaceae	Sequoia	1	+
	Sequoiadendron	1	+
	Metasequoia	1	0
	Taxodium	1	+
	Cryptomeria	1	+
	Cunninghamia	1	+
	Sciadopitys	1	+
	Athrotaxis	1	+
	Taiwania	1	+
Cupressaceae	Cupressus	3	+
	Chamaecyparis	5	+
	Thuja	4	0,+
	Thujopsis	1	0(+,28)
	Libroceđrus	4	+
	Pilgerodendron	1	+
	Callitris	5	+
	Neocallitropsis	1	+
	Tetraclinis	1	+
	Widdringtonia	1	+
	Fitzroya	1	+
	Diselma	1	+
	Juniperus	5	+
Podocarpaceae	Pherosphaera	1	0
	Phyllocladus	3	0
	Saxegothea	1	0
	Microcachrys	1	0
	Dacrydium	10	0
	Podocarpus	5	0
Cephalotaxaceae	Cephalotaxus	1	0
Araucariaceae	Agathis	3	0,+
	Araucaria	5	0,+
Taxaceae	Torreya	3	+(0,31)
	Taxus	3	+(0,31)
Welwitschiaceae	Welwitschia	1	0
Ephedraceae	Ephedra	1	0
Gnetaceae	Gnetum	1	0(+,14)

[a] 0 = rare or absent, + = distinct.

growth increment is characteristic of the species. Consequently, they suggested that the appearance of warts in tree species could be useful as a diagnostic feature.

The presence of warts in hardwoods has not been studied as systematically as in softwoods, and early observations were recorded using only a light microscope (17, 29). Any detail as minute as warts is difficult, if not impossible, to resolve with this instrument; therefore, these early data may be unreliable. Presented in Table II is a compilation of data from several sources summarizing the reported occurrence of a warty layer in angiosperm species. Only those observations made with an electron microscope are reported.

The majority of angiosperm species do not exhibit a distinct warty layer. One systematic exception is that warts are present in nearly all of the species examined in the order Myrtales, which includes families 135-144 in Table II.

Included in Table II are the observations of Scurfield, et al. (14) who used the scanning electron microscope to study the presence of warts and pit vestures in tracheary elements of 22 species distributed among the Gnetaceae and 16 angiosperm families. The authors concluded that the occurrence of these cell wall features can be placed in no sensible evolutionary context.

COMPOSITION

The chemical reactivity of the warty layer has been the subject of many investigations. Presented in Tables IIIA and IIIB is a summary of the literature on various treatments of the warty layer. Most workers agree that the warty layer is very resistant to chemical dissolution. A notable exception is Tsoumis (42) who argued that this structure could be extracted, or at least eroded, simply with hot water or with a series of hot organic solvents over

TABLE II

OCCURRENCE OF A WARTY LAYER WITHIN THE ANGIOSPERMS

No.[a]	Family	Genera	Species Examined	Appearance[b]	Reference
5	Magnoliaceae	Magnolia	2	0	(33,34)
		Liriodendron	1	0	(35)
7	Winteraceae	Bubbia	1	+	(14)
		Pseudowintera	1	+	(33)
8	Cercidiphyllaceae	Cercidiphyllum	1	0	(36)
30	Violaceae	Melicytus	1	0	(33)
35	Pittosporaceae	Pittosporum	2	0	(33)
39	Vochysiaceae	Vochysia	1	+	(14)
60	Dipterocarpaceae	Pentacme	1	+	(14,37)
		Monotes	1	+	(14)
		Parashorea	1	+	(37)
63	Malvaceae	Hoheria	2	0	(33)
65	Sterculiaceae	Triplochiton	1	0	(35)
66	Tiliaceae	Entelea	1	0	(33)
		Tilia	1	0	(31)
67	Elaeocarpaceae	Aristotelia	1	0	(33)
		Elaeocarpus	2	0	(33)
87	Meliaceae	Dysoxylum	1	0	(33)
		Entandrophragma	1	0	(35)
		Turraeanthus	1	0	(35)
90	Icacinaceae	Pennantia	1	0	(33)
93	Aquilifoliaceae	Ilex	1	0	(31,36)
99	Rhamnaceae	Discaria	1	0	(33)
101	Sapindaceae	Alectryon	1	0	(33)
		Dodonaea	1	+	(33)
102	Aceraceae	Acer	3	0	(31,35)
104	Hippocastanaceae	Aesculus	1	0	(31)
110	Corynocarpaceae	Corynocarpus	1	0	(33)
112	Coriariaceae	Coriaria	1	0	(33)
115	Leguminosae				
	--Mimosaceae	Acacia	2	+	(14,37)
		Plathymenia	1	+	(38)
		Sophora	1	0	(33)
	--Caesalpiniaceae	Gleditsia	1	0	(31)
		Goniorrhachis	1	+	(38)
		Gymnocladus	1	0,+	(14,31),(37)
	--Papilionaceae	Amburana	1	+	(38)
		Carmichaelia	1	s	(33)
		Notospartium	1	+	(33)
		Robinia	1	0,+	(35),(37)
117	Rosaceae	Prunus	1	0	(31)
		Rubrus	1	0	(33)
118	Saxifrageae	Ixerba	1	+	(33)
120	Cunoniaceae	Ackama	1	0	(33)
		Ceratopetalum	1	0	(35)
		Weinmannia	2	0	(33)

See end of table for footnote.

TABLE II (Continued)

OCCURRENCE OF A WARTY LAYER WITHIN THE ANGIOSPERMS

No.[a]	Family	Genera	Species Examined	Appearance[b]	Reference
121	Escalloniaceae	Carpodetus	1	0	(33)
		Quintinia	2	0	(33)
128	Hamamelidaceae	Altinga	1	+	(37)
		Liquidambar	1	0	(14,35,36)
135	Compretaceae	Terminalia	4	+	(14,37)
136	Myrtaceae	Eucalyptus	4	+	(13,14,33,37,39)
		Eugenia	2	+	(14,33,39)
		Leptospermum	2	+	(14,33)
		Lophomyrtus	1	s	(33)
		Metrosideros	3	+	(33)
		Neomyrtus	1	s	(33)
139	Lythraceae	Lagerstroemia	1	+	(14)
143	Sonneratiaceae	Sonneratia	1	+	(14)
144	Onagraceae	Fuchsia	1	s	(33)
157	Araliaceae	Pseudopanax	2	0,+	(33)
		Schefflera	1	0	(33)
158	Cornaceae	Cornus	1	0	(36)
		Griselinia	2	0,s	(33)
161	Nyssaceae	Nyssa	1	0	(31,36)
164	Rubiaceae	Calycophyllum	2	+	(8,14,37)
		Coprosma	8	0,s	(33)
168	Compositae	Cassinia	1	0	(33)
		Helichrysum	1	0	(14)
		Olearia	2	0	(33)
		Senecio	2	0	(33)
174	Ericaceae	Gaultheria	1	0	(33)
		Oxydendrum	1	0	(34)
177	Epacridaceae	Cyathodes	1	0	(33)
		Dracophyllum	1	0	(33)
182	Myrsinaceae	Myrsine	3	0	(33)
187	Oleaceae	Fraxinus	1	0,+	(35),(37)
		Olea	1	0	(33)
189	Apocynaceae	Aestonia	1	+	(14)
191	Loganiaceae	Geniostoma	1	s	(33)
198	Scrophulariceae	Veronica	1	0	(33)
203	Bignoniaceae	Catalpa	1	0	(35)
206	Myoporaceae	Myoporum	1	0	(33)
208	Verbenaceae	Avicennia	1	s	(33)
		Vitex	1	0	(33)
210	Plantaginaceae	Plantago	1	+	(17)
224	Piperaceae	Piper	1	0	(33)
226	Chloranthaceae	Ascarina	1	s	(33)
228	Monimiaceae	Hedycarya	1	s	(33)
		Laurelia	1	0	(33)
229	Lauracea	Beilschmiedia	2	0	(33)
		Sassafras	1	+	(34)
232	Proteaceae	Knightia	1	0	(33)
		Persoonia	1	+	(33)
234	Gonystylaceae	Gonystylus	1	+	(14)

See end of table for footnote.

TABLE II (Continued)

OCCURRENCE OF A WARTY LAYER WITHIN THE ANGIOSPERMS

No.[a]	Family	Genera	Species Examined	Appearance[b]	Reference
239	Santalaceae	Fusanus	1	0	(33)
243	Euphorbiaceae	Bridelia	1	+	(37)
245	Daphniphyllaceae	Daphniphyllum	1	0	(14)
250	Moraceae	Chlorophora	1	0	(35)
		Morus	1	0	(35)
251	Ulmaceae	Ulmus	5	0	(31,35)
		Celtis	1	0	(35)
252	Platanaceae	Platanus	1	+	(34,36)
254	Juglandaceae	Carya	1	0	(35)
257	Betulaceae	Alnus	1	s	(36)
		Betula	2	0,+	(35,36),(40)
258	Corylaceae	Carpinus	1	0	(34)
		Ostrya	1	0	(34)
259	Fagaceae	Castanea	1	0	(34)
		Castanopsis	1	+	(34)
		Fagus	4	+	(8,14,33,34,41)
		Nothofagus	5	0,+	(33,35)
		Quercus	8	0	(35)
260	Salicaceae	Populus	3	0	(33,35)
Monocotyledons					
	Liliaceae	Rhipogonum	1	0	(33)
	Pandaneae	Freycinetia	1	0	(33)
	Palmaceae	Rhopalostylis	1	0	(33)

[a] Numbers correspond to those of Metcalfe and Chalk (32) in listing Dicotyledon families.

[b] 0 = absent, s = possibly slight, + = distinct.

TABLE IIIA

TREATMENTS THAT REMOVE THE WARTY LAYER

Treatment	Action	Wood[a]	Reference
Hot (60°C) running water, 3 weeks	Removal or at least partial erosion of warty layer	G	(42)
Extraction with alcohol-benzene, alcohol, and water, Soxhlet apparatus, 12 hr	Removal of warts in one species, only slight change in another	G	(42)
50% Cold chromic acid	Warts dissolved slowly after 24 hr	G	(16)
72% H_2SO_4, room temperature 25 min	Warts could no longer be distinguished	Gr	(12)
78% H_3PO_4, room temperature 3 hr	Warts swell and then both they and the associated membrane dissolve	Gr	(12)
14% Nitric acid, 55°C, 1 hr; 14% nitric acid, 95°C, 1½ hr; then 3% NaOH, 95°C, 1 hr	Warts and vestures removed	A	(13)
30% H_2O_2 and glacial acetic acid (1:1), boiling	Warts dissolved after 4 hr	G	(16)
30% H_2O_2 and glacial acetic acid (1:1), 90°C, until sections were colorless	Dissolution of warts and membrane	Gr, A	(12,13)
30% H_2O_2 and glacial acetic acid (1:1), 60°C	Warty layer removed	G	(42,45)
NaOH solution, boiling 5 min, then cold chlorine water, 6 g/liter	Warts dissolved rapidly	G	(16)
Saturated chlorine water, 5 min; then 2% NaOH, room temperature; repeated until sections were colorless	Warts removed, but a wart-free membrane appeared to persist in some cells	Gr, A	(12,13)

See end of table for footnote.

TABLE IIIA (Continued)

TREATMENTS THAT REMOVE THE WARTY LAYER

Treatment	Action	Wood[a]	Reference
0.1N KOH extraction of chlorite holocellulose	All wartlike mounds removed	G	(43)
Solution containing 12.6% NaOH and 4.1% Na_2S, 60°C, 2½ hr	Warts may or may not be removed, a wart-free membrane sometimes occurred	Gr	(12)
Delignification by alkali digestion 120°C	The warty layer became chemically unstable and dissolved	G	(46,47)
Prolonged extraction in 5M urea	Membrane removed from cell lumens but not from pit chambers	G	(48)
White-rot fungi and some soft-rot fungi species	Warty layer eventually dissolved, warts decompose before the covering layer	A, G	(49,50)

[a]G = gymnosperm, A = angiosperm, r = reaction wood.

TABLE IIIB

TREATMENTS THAT DO NOT REMOVE THE WARTY LAYER

Treatment	Action	Wood[a]	Reference
Boiling water, 4 hr	Warts unchanged	G	(16)
Boiling alcohol, 4 hr; then boiling ether, 4 hr	Warts unchanged	G	(16,48)
Hot alcohol extraction	Resin droplets which can easily be confused with warts in pine were extracted, "true warts" remain intact	G	(52)
Chloroform extraction	No effect	G	(48)
Acetone extraction	No effect	G	(48)
Pyridine, boiling, 2 hr	Warts unchanged	G	(16)
Anhydrous ammonia, 145 psi, room temperature, 72 hr	Lumen surface became encrusted, warts still present	G	(53)
72% Sulfuric acid, 2 hr	Warts unchanged	G	(16)
98% Sulfuric acid, 2 hr	Warts unchanged	G	(16)
Butanol and HCl, boiling, 2 hr	Warts unchanged	G	(16)
80% Hydrofluoric acid, 12 days	Warty layer intact while carbohydrates were removed	G, Gr, A	(13,44)
80% Hydrofluoric acid, 160 hr; then 2% HCl, boiling gently	99.8% Lignin in residue, warty layer remained	A	(54)
30% H_2O_2, 98°C, 1½ hr	Warts unchanged	G	(16)
Sodium hypochlorite, cold, 8 hr	Warts unchanged	G	(16)
Acidified sodium chlorite solution, 75°C, 4 hr	Middle lamella completely removed, but warts not badly degraded	A, G	(37)

See end of table for footnote.

TABLE IIIB (Continued)

TREATMENTS THAT DO NOT REMOVE THE WARTY LAYER

Treatment	Action	Wood[a]	Reference
Delignification in sodium chlorite solution, pH 6.8 initially, 3 weeks, room temperature	72.1% Yield, remnants of wart structure remained	G	(43,55)
Neutral sulfite semi-chemical cooking liquor, 140°C, 60 min	88.7% Yield, warts unchanged but the areas between individual warts were laid bare	A	(56)
0.25M NaOH, boiling, 4 hr	Warts unchanged	G	(16)
17.5% NaOH, boiling, 4 hr	Warts unchanged	G	(16)
17.5% NaOH, 60°C, 2½ hr	Warts may undergo swelling but they were not dissolved; it was doubtful that the membrane was dissolved	Gr	(12)
5M Urea, boiling, 4 hr	Warts unchanged	G	(16)
1% Osmic acid, room temperature, 2 hr	No effect	Gr	(12)
2% Potassium permanganate, room temperature, 15 min	Deposition of granular material on wart surface; warts may undergo prior swelling	Gr, A	(12,13)
Groundwood pulping	Tracheids may be split open to reveal intact warty layer	G	(57)
Heating at 550°C	Membrane encasing the warts was destroyed; the warts remained exposed as crystal-like deposits (see footnote p. 21)	G	(51)
Steam under pressure, 150°C, 2 hr	Warts may swell but were not dissolved	A	(13)
2000-Yr natural degradation of submerged Viking ship	92.7% Lignin in remains, warts and warty layer still evident	A	(54)

See end of table for footnote.

TABLE IIIB (Continued)

TREATMENTS THAT DO NOT REMOVE THE WARTY LAYER

Treatment	Action	Wood[a]	Reference
Brown-rot fungi	Polysaccharides of the cell wall hydrolyzed; neither the warts nor the covering membrane appeared digested	A	(49,58)
Blue-stain fungi	No alteration of the warty layer	G	(59)
Xylanase attack after mild chlorite delignification	Warts were resistant	A	(60)

[a] G = gymnosperm, A = angiosperm, r = reaction wood.

a period of weeks. Here, however, the action may have been due more to physical erosion rather than chemical reaction or dissolution.

In addition to chemical treatments, the effect of a few microorganisms on the warty layer has also been explored. These latter treatments represent the action of complex enzyme systems. Commercial enzyme treatments and physical treatments have also been investigated and are reported in Tables IIIA and IIIB. In general, the results of the different treatments on wood were consistent over the wide range of species studied. This would seem to indicate that the reactivity and, therefore, the composition of the warty layer is similar from species to species.

Presented in Table IV are reported staining characteristics of the warty layer. While some investigators attributed a positive staining reaction to the presence of a certain chemical class in the warty layer, the staining reaction in reality may not have been that specific.

Workers have speculated on the composition of the warty layer by observing its chemical reactivity. Wardrop, et al. (16) stated that the warty layer had chemical reactions similar to those for lignin, but in addition showed staining evidence for the presence of protein, which is not normally found to any extent in the secondary wall. Dunning (43) speculated that warts in longleaf pine may be some combination of lignin and carbohydrate, since a mild oxidative treatment followed by an alkaline extraction was required for complete removal. Scurfield, et al. (12) concluded from ultraviolet absorption and chemical reactivity evidence that the warts, and perhaps any covering membrane, may have a lignin component. Côté, et al. (44) also implied that the warty layer contains lignin because warts were present in "lignin skeletons" created by hydrolysis of wood with 80% hydrofluoric acid. Côté

was unsure, however, whether or not the terminal lamella contained the same kind of lignin that occurred throughout the secondary wall (66).

TABLE IV

REAGENTS THAT STAIN THE WARTY LAYER

Reagent	Specificity[a]	Reference
Phloroglucinol-HCl	Lignin	(16)
Potassium ferrocyanide-$FeCl_3$	Protein	(16)
Methylene blue	--	(16)
2% OsO_4	--	(16,48)
Basic hydroxylamine	Uronic acid	(61)
Victoria Blue B	Hemicellulose containing uronic acid	(3,62,63)
$KMnO_4$	--	(64)
Uranyl acetate-lead citrate	--	(65)

[a]According to the reference quoted.

One indication that the wart material is different from cell wall lignin is that the wart structure remained more or less intact during some treatments that removed a major portion of the middle lamella and cell wall lignin. Côté and Day (37) showed that for both hardwoods and softwoods, a 4-hr digestion in acidified sodium chlorite at 75°C dissolved the middle lamella lignin but left the warty layer unchanged. Jayme and Azzola (56) reported no trace of damage to the wart structure of beech vessel members in a neutral sulfite semi-chemical pulp (87.7% yield, 16.5% lignin). Dunning (43, 55) reported that remnants of the wart structure remained in chlorite holocellulose of longleaf

pine (72.1% yield, 0.45% lignin). Berenzon and Bogomolov (46), however, found that during alkali delignification (kraft and soda pulping) the warty layer was chemically unstable and dissolved at 120°C.

An elemental composition of the warty layer has been reported only by Cronshaw, et al. (67). They isolated warts by dissolving the cell wall using the method of Pew (68), which consists of soaking wood sections in formaldehyde, macerating them in 72% H_2SO_4, dilution of the acid suspension with a 1:1 mixture of cellosolve and acetone, and subsequent centrifugation to sediment the wart residue. The presence of material in the residue similar in appearance to the warty layer was confirmed by electron microscopy. Microchemical analysis of the isolated warty layer from three different softwood species gave these results: carbon, 47.5-59.7%; hydrogen, 5.5-6.0%; oxygen, 32.4-39.4%; methoxyl, 3.3-7.3%; nitrogen, 0.6-2.1%; and phosphorus, 0.0-0.3%. There are at least two reasons why these values cannot be regarded as absolute. There is a high probability that material other than warts, such as insoluble middle lamella or secondary wall lignin, was present in the residue. Also, the isolated wart material was probably altered chemically by such a harsh extraction.

Wardrop, et al. (48) reported a chemical-physical technique that was successful in isolating an osmiophilic layer with a wartlike texture which lined the lumen. They cut 20-μm longitudinal sections from osmium-stained blocks and treated them in a mechanical disintegrator. Fragments of the osmiophilic membrane could be separated because of their dark color among the cell debris. The membrane was isotropic when viewed with polarized light and was thus assumed not to contain highly-oriented cellulose. This method of isolating the warty layer did not yield any large quantity of material (16). Therefore, subsequent analysis was not attempted.

It is known that warts occur in some species without an associated amorphous layer. There is some feeling that the composition of the individual warts is different from that of any amorphous layer, when present. This idea was based on the different response of these two structures to various physical and chemical treatments. In UV light (216 nm) the warts were characterized by high absorbing properties while the membrane displayed a very weak absorbance (16). (This result, however, could have been due to a concentration effect rather than any difference in composition.) Warts in vessel elements of beech latewood were more resistant to neutral sulfite semichemical cooking liquors than the membrane which was noticeably attacked (56). Tsoumis (42), on the other hand, showed that warts were destroyed during prolonged solvent treatments, while the region between the warts remained undissolved. Knudson and Williamson (51) reported that on heating Douglas-fir wood to 550°C, membranes encasing the warts on pit tori and cell walls were destroyed, leaving the warts exposed as crystal-like deposits.* White-rot fungi and also some soft-rot species were found to dissolve both components, but the warts appeared more readily decomposed than the layer, in that small craters sometimes occurred during the first stages of enzymatic dissolution (49, 50, 69). Scurfield and Silva (12) showed with chemical reagents that wart dissolution does not necessarily involve simultaneous dissolution of the associated membrane. They concluded that "warts and membrane apparently differ in chemical composition."

Several workers have pointed out similarities between warts and the pit vestures found in the vessel members of some hardwood species (3, 13, 14, 37-39).

*Discrepancies here are that warts have not been previously reported on pit tori of any species and warts on the cell wall of Douglas-fir are rare or entirely absent (6, 35).

Schmid and Machado (38) acknowledged these similarities but felt that the two structures have different origins in the developing cell. Scurfield, et al. (14), however, concluded that there seems to be no adequate reason for distinguishing vestures from warts other than on the grounds of the greater structural complexity of the former.

In another study, Cronshaw, et al. (67) have shown that the warty layer is morphologically very similar but chemically different from the pollen sexine at the outer portion of the exine of pollen grains.

ORIGIN

The origin of the warty layer in the developing wood cell is a subject of controversy. Liese (4) reported that the layer consists solely of dead protoplasmic material deposited on the lumen surface at the end of cell differentiation. According to this view, the warty layer comprises two protoplasmic membrane residues, the plasmalemma and vacuole tonoplast, with fragments of cytoplasmic debris trapped between to form the warts.

Wardrop and Davies (64) found evidence in developing cells that wartlike protrusions are formed as part of, and continuous with, the last deposited secondary wall layer, S3. At cell death the cytoplasmic membranes retract and ultimately dry onto the lumen surface, enclosing the remaining organelles which form spherical bodies on top of wartlike, cell wall protrusions. Thus, according to these authors, the complete wart structure consists of localized cell wall thickenings with a covering of residual cytoplasmic components.

Several workers have suggested different explanations for the origin of the warty layer solely from observations of wart morphology in mature wood cells and without examining developing tissue. According to Jurbergs (52),

who studied four pine species, the nonuniformity in the distribution of warts and their preferential accumulation at certain sites are evidence that these structures are not produced by the still-organized cell protoplast. He interpreted the observations to indicate a random process of wart deposition, perhaps by sedimentation of decomposed protoplasts during translocation of the latter from the mature cell.

In contrast, Frey-Wyssling and Muhlethaler (70) concluded that a morphogenic factor is active during wart formation in addition to passive degradation of the cell contents. Their conclusion was based on the relationship that exists between warts and dentate ray tracheids in pine and between warts and vestured pits in some hardwood species.

Esau (in 5) raised a good question concerning the supposition that the warts consist totally or partially of cytoplasmic organelles that settle on the cell wall after differentiation is complete: "I am disturbed that these organelles appear like little pebbles which have settled down. Actually, when they die and dry up, they will not be recognizable as little bodies, will they?"

Cronshaw (65) also could not accept the cytoplasmic debris theory for the origin of the warty layer, explaining that this view is difficult to reconcile with the fact that the conifer tracheids do not "dry out" until heartwood formation, many years after formation of the warty layer. He also cited the taxonomic significance of the warty structure as evidence against this theory.

Upon detailed study of pine cambial tissue, Cronshaw (65) found that the wart structure was developed external to the plasma membrane during the final stages of cell differentiation. It formed the innermost layer of the cell

wall and was distinct from the S3 layer. Later, the protoplast degenerated and disappeared from the cell. Therefore, according to this view, the warty layer is neither cytoplasmic remains nor part of the S3 layer. Rather, it is a structure elaborated by the living cytoplasm in its final role before disintegration.

Cronshaw identified two possible cytoplasmic systems which could contribute precursors and enzymes from wart formation. Both the Golgi bodies (and Golgi body-derived vesicles) and the endoplasmic reticulum were evident in the receding cytoplasm of cells during the later stages of differentiation. Elsewhere (71-73), it is argued that these two organelles are probably also functional in the synthesis of plant cell wall materials.

If residual cytoplasm is not dried onto the cell wall as earlier workers have believed, what then is its fate? Cronshaw's explanation was that cytoplasmic contents are depolymerized and eluted out of the cell by the transpiration stream as the tracheids become functional for water conduction. This suggestion is consistent with the results of Merrill and Cowling (74) and Grozdits and Ifju (75) who observed that there was a sharp decrease in nitrogen content within the last-formed growth increment of several woody species and that samples taken from other annual increments had a remarkably low nitrogen content.

Kutscha (19), in support of Cronshaw's work, also found that the warty layer is deposited apparently external to the plasmalemma following secondary wall formation. He observed continuity between the tips of the conelike warts and various membrane-bound vesicles or dark-staining particles in the cytoplasm. Wart formation in compression wood apparently follows the same course (19).

Despite Cronshaw's convincing report, his work has been overlooked or disregarded in several recent reviews and texts (e.g., 76, 77) which continue to explain the origin of the warty layer as "cytoplasmic debris and possibly localized thickening of the S3 layer." Such disregard would seem unwarranted and misleading.

Scurfield and Silva (12), considering the origin of the warty layer, speculated that warts constitute a replica of invaginations of the plasma membrane through which wall materials are actively secreted at the time of cell death. Warts might then be regarded as consisting of something less than completely elaborated wall constituents plus the products of protoplast autolysis, which are reported by Scurfield to consist largely of phenolic substances (9). This speculation is not inconsistent with known properties of the warty layer, but it does lack verification.

SIGNIFICANCE

The presence of the warty layer lining the lumen surface of wood cell walls may have some effect on gross wood properties both in nature and in the subsequent processing of woody tissue. The warts provide increased lumen surface area and surface roughness. Also, this layer, if lignin is present, probably has a lower hydrophilicity than an exposed, microfibrillar S3 layer. It is, therefore, reasonable to propose that these cell wall properties, due solely to the presence of the warty layer, likely influence the flow of the aqueous transpiration stream in conducting sapwood of a living tree or the penetration of liquids through the cells in cut wood.

Several investigators (78-80) have suggested that the freer movement of liquids through aspirated bordered pits in softwoods is at least partially

due to the wart structure in the pit chamber. If an aspirated torus abuts a warted border, the adhesion cannot be as strong as the contact between the same torus and a smooth border. As a result, the pit is less firmly sealed (Fig. 2). While this mechanism for increased permeability does not completely explain all the permeability differences observed among conifers, it may play an important part (78). Such an effect is not as great as might be expected because in many conifer species warts are very sparsely distributed near the pit aperture (78).

Since the warty layer appears to be quite chemically resistant, and since it is the terminal lamella of the cell wall, it is not unreasonable to propose that this structure may act as a barrier to the penetration of liquids from the cell lumen into the wall. For the pulp and paper industry, this would be an important consequence because pulping liquors must diffuse through the cell wall in order to dissolve intercellular substances and thereby free individual fibers. If the warty layer does, in some instances, act to curtail penetration, then such information should affect the papermaker's choice of wood species or pulping procedure. This concept that the warty layer may act as a barrier has been briefly considered before (6, 81), but there apparently has been no published investigation designed specifically to ascertain any effect the warty layer might have on penetration of liquids into the wood cell wall.

Another possible consequence of the presence of warts in wood tissue emerges in the production and processing of dissolving pulp. The resistance of the warty layer to chemical dissolution may contribute to the difficulties in filtering solutions of cellulose acetates and viscoses and may also be responsible for haziness in resulting films or weakness in resulting fibers. Sperling and Easterwood (82) identified two classes of characteristic gel

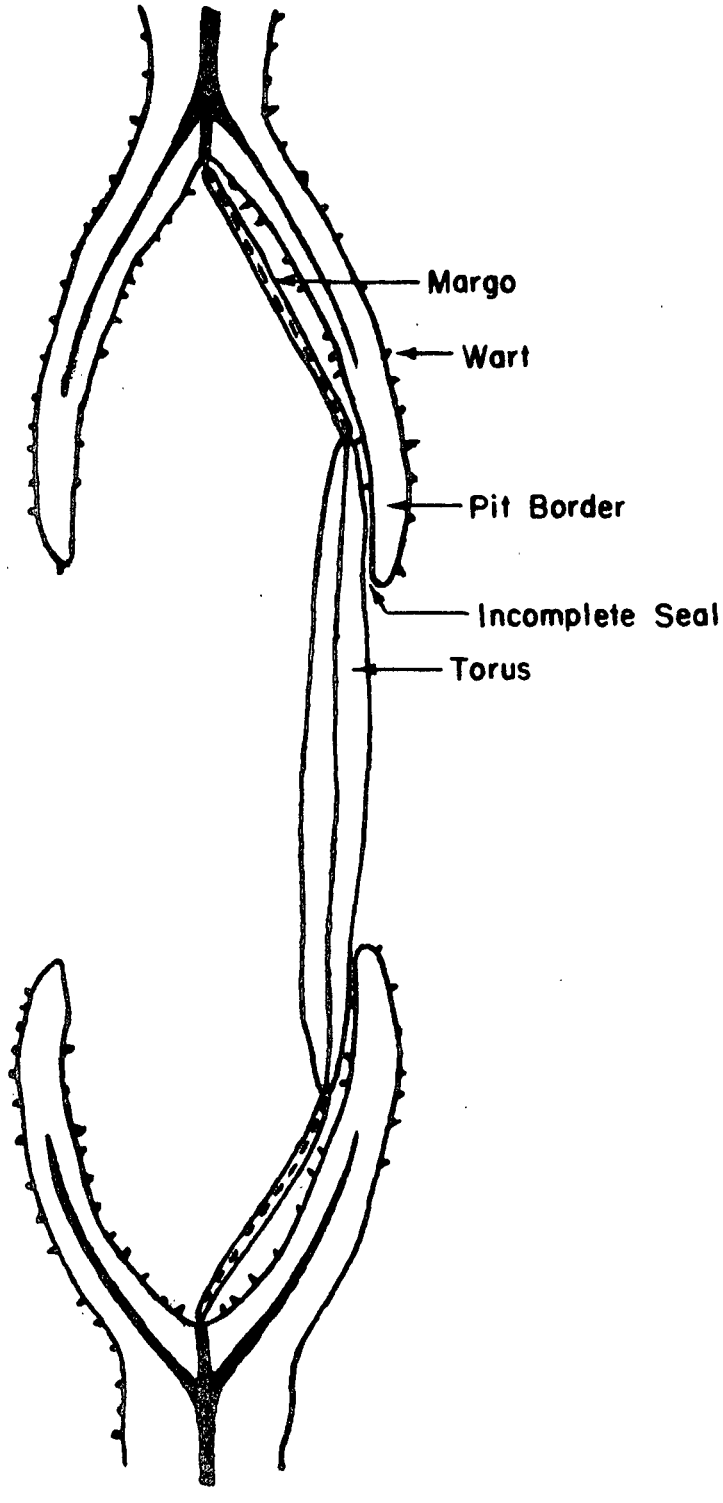


Figure 2. Diagram of an Aspirated, Intertracheid Bordered Pit of a Softwood; for an Actual Micrograph, see Cote (29), Fig. 5

particles from material blocking filters and causing solution haze in cellulose acetates: those over 15 μm and those quite a bit smaller. The predominant characteristics observed among the small gel particles was a nearly monodisperse spherical- or potato-shape and a mean diameter of about 0.3 μm in dispersion (light-scattering) and 0.1 μm dry (electron microscopy). These measurements are quite consistent with the wart size. Chemical analyses showed these small particles to contain cellulose and xylan.

An analogous study on wood pulp viscose yielded similar results in regard to number, size, monodispersity, and swelling characteristics of the small gel particles (83). A major difference, however, was a lack of xylan in the composition. Only half of the minute viscose insolubles was carbohydrate material, probably cellulose. The other half was not identified.

Jurbergs (52), working in the same laboratory, felt that the source of the tiny gels in cellulose solutions was in the original pulp. The most conspicuous features of this size present in large numbers in wood are warts. The fine gels and resulting haze were also found, however, in dissolved pulps from cotton linters which probably do not have warts (23). But, yield of fine gel from this source was considerably lower.

It is questionable whether the warts in wood can survive the harsh pulping and bleaching procedures required for production of dissolving pulps. Jurbergs (52) apparently did not consider this point as a prerequisite to his hypothesis.

OBJECTIVES AND APPROACH OF THE THESIS

There were two primary objectives of the thesis. The first was to determine the origin of the warty layer in the differentiating cell. The second was to determine the chemical composition of this structure as it exists in

mature wood. As is implied in the Historical Review, both of these areas have been investigated in previous work, but there is still a great deal of controversy surrounding both points.

In order to study the origin of warts, developing tissue was examined in ultrathin section and from replicas of the interior surface of the differentiating cell wall. The latter approach is unique to the study of warty layer development.

The composition of warts in mature wood was studied by using the electron microscope to monitor the results of various chemical, physical, fungal, and enzymatic treatments on the warty layer. In conjunction with selected chemical treatments that were found to dissolve the warts, analyses were performed to identify residual as well as extracted material.

There were two secondary objectives of the thesis. The first was to determine, if obvious under limited conditions, any effect of the presence of the warty layer on the removal of lignin from the cell wall. The second was to determine any evolutionary significance of the warty layer in wood.

The possible effect of a warty layer on pulping of woody tissue was studied briefly by comparing initial delignification rates of two softwood species with different lumen linings.

A number of hardwood species were examined to determine if the presence of the warty layer can be placed in any logical evolutionary context. A special effort was made to correlate the presence of a warty layer with the occurrence of any other cell wall feature of suspected evolutionary significance.

Balsam fir was chosen as the major source of experimental material because it has a very prominent warty layer and it is locally available. Also balsam fir is a commercially important pulpwood in the Northeast, the Lake States, and in eastern Canada.

MATERIALS AND METHODS

SOURCE OF THE WOOD

The source of the cambial tissue examined in this investigation was an erect balsam fir [Abies balsamea (L.) Mill.], 12-inch dbh, growing in a mixed stand north of Black Creek, Wisconsin. Samples were taken on June 12, and July 13, 1972, according to the procedure described in Appendix I.

Increment core samples (11 mm in diameter) of mature sapwood were taken on August 12, 1971 from another erect balsam fir, 8-inch dbh, growing near Tomahawk, Wisconsin. After extraction from the tree, these samples were immediately sealed in black rubber tubes and stored in a freezer until needed.

Mature stem and root xylem were also cut from a normal 3-yr-old balsam fir seedling grown in a greenhouse at The Institute of Paper Chemistry.

Thin, acetone-extracted, earlywood wafers of loblolly pine (Pinus taeda L.) were obtained from a supply left from the completed research of Albrecht (84). The wafers were tangential sections, approximately 0.5 mm in thickness.

Samples of hardwood species to be studied were cut from sapwood of dry wood blocks from a collection at The Institute of Paper Chemistry. The following species were examined:

American hornbeam (Carpinus caroliniana Walt.)

eastern hophornbeam [Ostrya virginiana (Mill.) K. Koch]

coachwood (Ceratopetalum apetalum D. Don)

golden chinkapin [Castanopsis chrysophylla (Doug.) A. DC]

American chestnut [Castanea dentata (Marsh.) Borkh.]

American beech (Fagus grandifolia Ehrh.)

Mediterranean beech (F. orientalis Lipsky)

European beech (F. sylvatica L.)

negro-head beech (Nothofagus moorei J. H. M.)

sassafras [Sassafras albidum (Nutt.) Nees]

cucumber magnolia (Magnolia acuminata L.)

southern magnolia (M. grandiflora L.)

American sycamore (Platanus occidentalis L.)

sourwood [Oxydendrum arboreum (L.) DC.]

ELECTRON MICROSCOPY

Both replica and ultrathin sectioning techniques were used to prepare material for examination with the transmission electron microscope (TEM). Surface replicas of wood sections were prepared by the direct-carbon method outlined by Côté, et al. (85) using the procedure and apparatus described by Dunning (43). Platinum, applied at an angle of 45° from 8 cm, was employed to cast a shadow parallel with the axis of the tracheids. Carbon and metal coatings were applied in a Denton Vacuum Model DV502 vacuum evaporator. Metalized wood surfaces were backed with polystyrene (Appendix VI) and the wood was dissolved with 72% H_2SO_4 (18 hr) followed by 10% chromic acid-10% nitric acid (1:1) (4 hr). The polystyrene was then dissolved with benzene from the replicas, which were supported on 100-mesh nickel grids precoated with collodion and carbon for TEM examination. Replicas and ultrathin sections were examined with an RCA Model EMU-3F TEM at 50 kv.

Samples for direct observation with the scanning electron microscope (SEM) were oven-dried at 60°C and affixed to standard specimen pedestals with transfer adhesive tape (3M no. 465). They were then coated omnidirectionally with

approximately 20 nm each of carbon and 60:40 gold-palladium to prevent specimen charging in the electron beam. Samples were examined with a JEOL JSM U-3 SEM at 15-25 kv.

CAMBIAL TISSUE

Two forms of cambial samples, radial slices and blocks, were cut from the 12-inch dbh balsam fir tree by the procedure described in Appendix I. The radial slices were processed for eventual TEM study of developing cells. They were first fixed with either permanganate or one of two aldehyde-mixture schedules outlined in Appendix II. They were then dehydrated and embedded with an epoxy resin by one of the procedures in Appendix III. Ultrathin cross sections of the embedded cambial tissue were cut about 60 nm in thickness with a diamond knife on a Sorvall MT-2 ultramicrotome. The sections were picked up on the previously described grids and stained with lead citrate or potassium permanganate or both in sequence by the procedure in Appendix IV.

The block-size cambial samples were prepared so as to permit inspection of the inner, radial surface of the developing cell wall. Cytoplasmic contents were not chemically fixed. The blocks were embedded with collodion according to Appendix V, and then sectioned radially at about 100 μm with a sliding microtome. The collodion embedment was dissolved with ether-alcohol (1:1), and the sections were then dried from ether and replicated from the TEM or coated for SEM study.

MATURE WOOD

The frozen increment cores of fir sapwood were allowed to thaw and become water-saturated. They were then trimmed into blocks and sectioned radially at about 100 μm with a sliding microtome. After subjection to various treatments,

replicas were prepared for TEM study to determine the effect of the treatment on the warty layer and other ultrastructural wood features.

Treated and untreated loblolly pine wafers as well as untreated stem and root tissue from a balsam fir seedling were also prepared for examination with the TEM.

Hardwood samples were saturated in water and sectioned radially at about 150 μm with a sliding microtome. After drying and metal coating they were examined with the SEM. Surface replicas were made of selected samples for more detailed examination with the TEM.

TREATMENTS

Sections of balsam fir sapwood were subjected to chemical, enzymatic, fungal, and physical treatments in an effort to dissolve or otherwise remove the warty layer. Unless otherwise specified, the treated tissue was from the never-dried increment cores and was in the form of radial sections, 100 μm thick. Only the surfaces of the treated sections were examined with the TEM, but Albrecht (84) has shown that penetration and reaction is homogeneous in sections even five times as thick.

CHEMICAL TREATMENTS

Unless otherwise specified, all chemicals used were reagent grade.

1. Sequential extraction in a Soxhlet apparatus: benzene-ethanol (2:1), 6 hr; ethanol, 6 hr; water, 2 hr.
2. Dioxane extraction, room temp., 3 hr to 6 days; and 60°C, 24 hr.
3. Dimethylformamide, 125°C, 27 hr.
4. Phenol, 90°C, 21 hr.

5. 78% Phosphoric acid, room temp., 3 hr.

6. 10% Chromic acid-10% nitric acid (1:1), room temp., 1½ hr.

7. 72% H_2SO_4, with and without polystyrene backing on wood section (Appendix VI), room temp., ½ and 35 hr.

8. Dimethylsulfoxide (DMSO)-0.1 or 0.5% HCl, 150°C, 1 to 5 hr.

9. Hydrogen peroxide and acetic acid, varying concentrations and proportions, room temp., 45°C or 90°C, 2 hr.

10. Chlorite delignification (86), 4.5% sodium chlorite (technical grade), pH 4.0 with acetic acid, dark, room temp., 4 weeks, wood treated as blocks.

11. 12.5% Sodium hydroxide, 4.1% sodium sulfide, 60°C, 2½ hr.

12. 17.5% Sodium hydroxide, 60°C, 2½ hr.

13. 0.1N Potassium hydroxide, 90°C, 2 hr.

14. 0.25% Digitonin (membrane-dispersing agent, product of Nutritional Biochemical Corp.) in 95% ethanol, room temp., 18 hr to 11 days.

15. 5.0M Urea, room temp., 3 hr to 6 days.

16. 4-Methylmorpholine 4-oxide [carbohydrate and lignin solvent (87)], 90°C, 22 hr.

17. Cadoxene [cellulose solvent (88)], room temp., 35 hr and 4 days.

18. NCS Tissue solubilizer (quaternary ammonium base in toluene, product of Amersham/Searle), 50°C, 22 hr.

The action of the four following treatments was studied more extensively:

1. DMSO, 150°C, 1 to 15 hr, 10-ml DMSO.

2. Dioxane-0.5% HCl, 70°C, 1 to 56 hr, 15-ml reagent.

3. 30% Hydrogen peroxide, room temp., 2 to 14 days, 25-ml reagent, wood submerged under an inverted funnel to keep sections below surface.

4. 3% Peracetic acid with 0.5% hydrogen peroxide, 60°C, 1-1/3 to 25 hr, 15-ml reagent.

For these four treatments, batches of 20 fir sections (about 50 mg) were treated. Loblolly pine wafers were also treated with peracetic acid. The change in ultrastructure as a result of the treatments was examined with the TEM. In early work, fir sections were extracted before treatment as specified in Chemical Treatment No. 1. This prior extraction had no effect on subsequent reactivity of the warty layer; therefore, the procedure was discontinued.

Yield calculations were based on the dry weight of the wood before and after treatment. The wood was vacuum-dried for 2 hr at either 105°C or 60°C, allowed to cool in a vacuum desiccator over Drierite, and weighed. Klason lignin determinations were made on the treated sections by using 1/20th proportions of those specified in TAPPI Standard T 13 m-54. Apparent carbohydrate portion was determined by difference. The treatment solutions containing dissolved wood components were subjected to various analyses described later. When desired, the DMSO and dioxane-HCl treatment solutions were concentrated into a syrup by freeze drying with a New Brunswick Freeze Dryer. The concentrates were then redissolved in small amounts of DMSO or dioxane.

ENZYMATIC TREATMENTS

The action of various enzymes on the ultrastructure of fir wood was explored. The known specificity of enzymes could make them a more desirable treatment than most chemical reagents. Unless otherwise stated, the fir sections were treated in 10 ml of enzyme solution. Before use in treatment, the activity of each enzyme was checked using the substrate and conditions suggested by the supplier. The enzyme, supplier, reported activity, buffer, and conditions are as follows:

1. Lipase 448 (Nutritional Biochemicals Corp.), 40 units/ml, 0.05M TRIS buffer, pH 7.8, 37°C, 18 hr.

2. Ribonuclease (Worthington Biochemical Corp.), 2700 units/ml, phosphate buffer, pH 7.0, room temp., 4 to 20 days.

3. Trypsin (P-L Biochemicals, Inc.), 10,000 units/ml, 0.012M $CaCl_2$, 0.05M TRIS buffer, pH 7.8, room temp., 2 hr.

4. Pepsin (Worthington Biochemical Corp.), 2,500 units/ml, 0.01M HCl, room temp., 2 hr.

5. Lysozyme (Mann Research Laboratories), 9,000 units/ml, phosphate buffer, pH 7.0, room temp., 2 hr to 20 days.

6. Hemicellulase (Nutritional Biochemicals Corp.), 5 units/ml, citrate buffer, pH 4.5, 45°C, 2 hr to 28 days.

7. Peroxidase (Nutritional Biochemicals Corp.), 3,000 units/ml, 0.003% to 0.3% H_2O_2, 0.01M phosphate buffer, pH 6.0, room temp., 8 to 95 hr; enzyme treatment on untreated wood and in combination with an alkali pre- or posttreatment (0.1N KOH, 90°C, 2 hr).

8. Polyphenol oxidase (P-L Biochemicals, Inc.), 500 units/ml, 0.2M phosphate buffer, pH 6.5, room temp., 18 hr; enzyme treatment on untreated wood and in combination with an alkali pre- or posttreatment (0.1N KOH, 90°C, 2 hr).

9. Pectinase (Nutritional Biochemicals Corp.), 2 mg/ml (1 g of enzyme reduces the viscosity of 1,500 g of pectin by 50% in 15 min), 0.05M acetate buffer, pH 3.7, 45°C; enzyme treatment on:

    a) never-dried fir section, 2 hr to 28 days.

    b) fir section infected with white-rot fungus (Trametes suaveolens) for 8 weeks, enzyme treatment 18 hr.

    c) loblolly pine wafer, 18 hr.

    d) 0.5 g fir chlorite holocellulose (Chemical Treatment No. 10), 25-ml enzyme solution, 18 hr.

Before the pectinase treatment of fir chlorite holocellulose, the enzyme was dialyzed with a regenerated cellulose membrane to remove the glucose known to be present in the preparation (89). After enzymatic hydrolysis of the delignified fir, the supernatant was decanted and boiled to denature the enzyme, which was subsequently sedimented by centrifugation at 12,100 × g for 20 min. The solution was then batch-treated with Amberlite IR 120 CP cation

exchange resin to convert the buffer to acetic acid, followed by concentration to dryness in a rotary vacuum evaporator at 55°C. (Water was added during the evaporation step until the pH of the solution was at least 6 to ensure that the acetic acid was azeotropically removed before concentration to complete dryness.) Two control solutions, the supernatant of delignified fir wood in buffer without enzyme and the enzyme solution alone, were worked up in a similar fashion.

FUNGAL TREATMENTS

All white-rot fungi used were purchased from the American Type Culture Collection, Rockville, Maryland. (Numbers with the fungi refer to the ATCC strain numbers.) Before wood was added, the fungi were cultured aseptically in shake flasks containing 50 ml of one of two different media, asparagine-glucose (asp.-glu.) or malt (see Appendix VII). Firwood in the form of 100-μm radial sections was sterilized by autoclaving and then introduced to the cultures after the rapid growth phases of the fungi had occurred (1-3 weeks). The white-rot fungi, the culture media, and the treatment durations are given below:

1. Daedalea unicolor 9405, asp.-glu., 2 to 26 weeks.
2. Polyporus anceps 13242, asp.-glu., 2 to 26 weeks.
3. Schizophyllum commune 9418, asp.-glu., 2 to 26 weeks.
4. Trametes suaveolens 9417, asp.-glu. and malt, 2 to 14 weeks.
5. Poria subacida 12241, asp.-glu., 2 to 10 weeks.
6. Polyporus versicolor 12679, asp.-glu., 2 to 10 weeks.

After treatment, the wood sections were scraped with a dissecting needle to remove the fungus from the sample surface before preparation for TEM study.

THERMAL TREATMENT

The thermoplastic properties of the warty layer were investigated by examining replicas of water-saturated wood that had been heated in an autoclave at 15 psi steam (121°C) for ½ hr and 2½ hr.

PHYSICAL ISOLATION METHODS

Several attempts were made to physically isolate the warty layer.

1. Firwood in the form of 20-µm cross sections and 100-µm radial sections was ultrasonicated in water, 0.1N KOH, or pectinase solution at 300 kc/sec for 2 hr at 30°C in a General Electric ultrasonic generator.
2. Wood blocks were macerated with a meat grinder and then pulverized with a pestle and mortar containing fine sand. The suspended wood particles were fractionated according to size by differential centrifugation using increasing speeds up to 37,000 × g for 15 min with the Sorvall Model RC 2-B centrifuge.
3. The surfaces of radial wood sections were embedded in molten polystyrene (Appendix VI). As much wood as possible was then scraped away using a dissecting needle while observing with a stereomicroscope. The polystyrene was then dissolved to free the minute, embedded wood fragments.

The results of the physical isolation methods listed above were evaluated with the TEM and SEM.

ANALYSIS OF TREATMENT SOLUTIONS AND PHYSICALLY ISOLATED WOOD FRAGMENTS

The pH values of extraction solutions were determined with a Beckman Zeromatic pH meter with glass electrode. Where indicated, the ultraviolet absorbance spectra of treatment solutions were determined with a Cary 15 spectrophotometer.

Centrifugation of selected DMSO treatment solutions was accomplished with the Sorvall Model RC 2-B centrifuge with rotor SS-34 at 13,000 rpm (20,200 × g) for 20 min. Ultracentrifugation was done with the Beckman Model L-2 ultracentrifuge with rotor SW39L at 30,000 rpm (75,000 × g) for 90 min. These procedures were carried out to discover if perhaps the warts were freed from the cell wall but not dissolved by the treatment. The sedimentation coefficient of the material dissolved in the DMSO treatment was determined by sedimentation velocity in a Beckman Model E ultracentrifuge. The sample was rotated at 56,100 rpm (230,000 × g) for times up to 25 min.

Refractive indices of the DMSO treatment solutions were measured with an Abbe refractometer at 18.8°C.

Thin-layer chromatography was used to fractionate materials in the pectinase hydrolyzate of chlorite-delignified firwood. Spots of 2 µl of the concentrated hydrolyzate were applied on precoated Kieselguhr plates buffered with 0.05M sodium acetate and developed with four repetitions with Solvent I, ethyl acetate-isopropanol-water (8:2:1). Controls and reference compounds were run simultaneously. Detection was accomplished by spraying with anisaldehyde reagent (Appendix VIII).

The technique of descending paper chromatography using Whatman No. 1 paper was employed to fractionate both carbohydrate materials and lignin materials in the treatment solutions. To develop carbohydrate materials, two different solvent systems were employed, namely, either Solvent II, ethyl acetate-acetic acid-formic acid-water (18:3:1:4) alone or Solvent III, ethyl acetate-pyridine-water (8:2:1) to move the neutral sugars away from the acids, followed by Solvent II. Spots of 2 µl (about 50 µg of dissolved material) were applied. Controls and known reference compounds were run simultaneously. Chromatograms were detected with p-anisidine hydrochloride (Appendix VIII).

The paper chromatographic fractionation of nonvolatile, low molecular weight lignin material was attempted using Solvent IV, butanol-pyridine-water (10:3:3). The paper was spotted with 4 µl of the concentrated treatment solutions. Developed components were detected with either 2,4-dinitrophenylhydrazine or diazotized p-nitroaniline (Appendix VIII).

Gas-liquid chromatography was used in an attempt to isolate semivolatile components extracted from wood in the DMSO and dioxane-HCl treatments. The instrument used for the fractionation was a Varian Aerograph series 1400 gas chromatograph fitted with a 6-ft column of 10% Carbowax 20M on a support of Chromasorb W, AW-DMCS, 60/80 mesh. The operating conditions were as follows: injector temp., 210°C; column temp., 150°C for 16 min, then programmed to increase at 10°/min to 210°C where the temperature was held; detector temp., 280°C; helium carrier gas flow was about 75 ml/min. The unit was equipped with a flame ionization detector. The sample injection volume was 5 µl of the unconcentrated treatment solutions. The extraction solvents alone were run as controls. Also, the retention times of known compounds, phenol, guaiacol, and furfural, were determined.

Gel-filtration chromatography was used to fractionate treatment solutions by molecular weight. Sephadex G-25 and DMSO were used as the gel-eluent system. [Lundquist and Wesslen (90) have reported that, with this system, 21 lignin model compounds ranging in molecular weight from 124 to 512 were eluted in approximate order of decreasing molecular weight.] The column was 1 cm in diameter and 75-cm high. The boundaries of the included volume of the column were determined by eluting acetone and a high molecular weight lignin sample prepared according to Pepper, et al. (91). DMSO and dioxane-HCl treatment solutions were freeze-dried and redissolved in a small amount of DMSO. One half milliliter of the concentrated solution was loaded on the column. The flow rate was maintained at about 0.8 ml/min. The column was constantly monitored by recording absorbance of the eluent at 280 nm with a 1-mm flow cell installed in a Cary 15 spectrophotometer. Pure DMSO in a stationary 1-mm cell was used as a reference.

Wood fragments isolated by scraping polystyrene-embedded sections were analyzed for elemental composition by monitoring characteristic x-rays emitted under electron bombardment of the sample. An EDAX x-ray detector and analyzer was used in combination with the SEM in this analysis. This instrument can detect characteristic x-rays for elements with atomic number greater than ten.

RESULTS AND DISCUSSION

STRUCTURE OF BALSAM FIR

Wood, as a natural product, has a characteristic morphology at all levels of examination. Figure 3 shows a corner view of a block of balsam fir, a softwood. About 95% of the volume of balsam fir consists of one type of cell, the longitudinal tracheid (77), which is a relatively long, prismatic element with tapering, closed ends. The warty layer covers the inner cell wall surface of these cells as shown in Fig. 4. (In this work, the tracheid axis in all electron micrographs of replicas is parallel with the direction of the white shadows.) Figure 5 depicts the warty layer in an ultrathin radial section of the cell wall. Warts also line the cavities of bordered pits, but the distribution is sparse near the aperture (Fig. 6). Warts occur in the tracheids of juvenile and adult wood in the roots, trunk, and branches. They were never observed in xylem ray cells or in any cells of the phloem.

The warts in balsam fir appear as blunt cones with an average size of about 0.1 μm in diameter at the base and about 0.2 μm in height. They are slightly smaller in latewood cells and in bordered pit cavities. Their frequency averages about 18 warts/μm^2 in earlywood and slightly less in latewood. Because of their protrusion from the cell wall, the total inner cell wall surface area is about twice what it would be if no warts were present. From average tracheid dimensions and some reasonable geometric approximations, it was calculated that there are about eight million warts in a typical, mature earlywood tracheid. The volume of the warts plus accompanying amorphous encrustant makes up about 2% of the total cell wall volume.

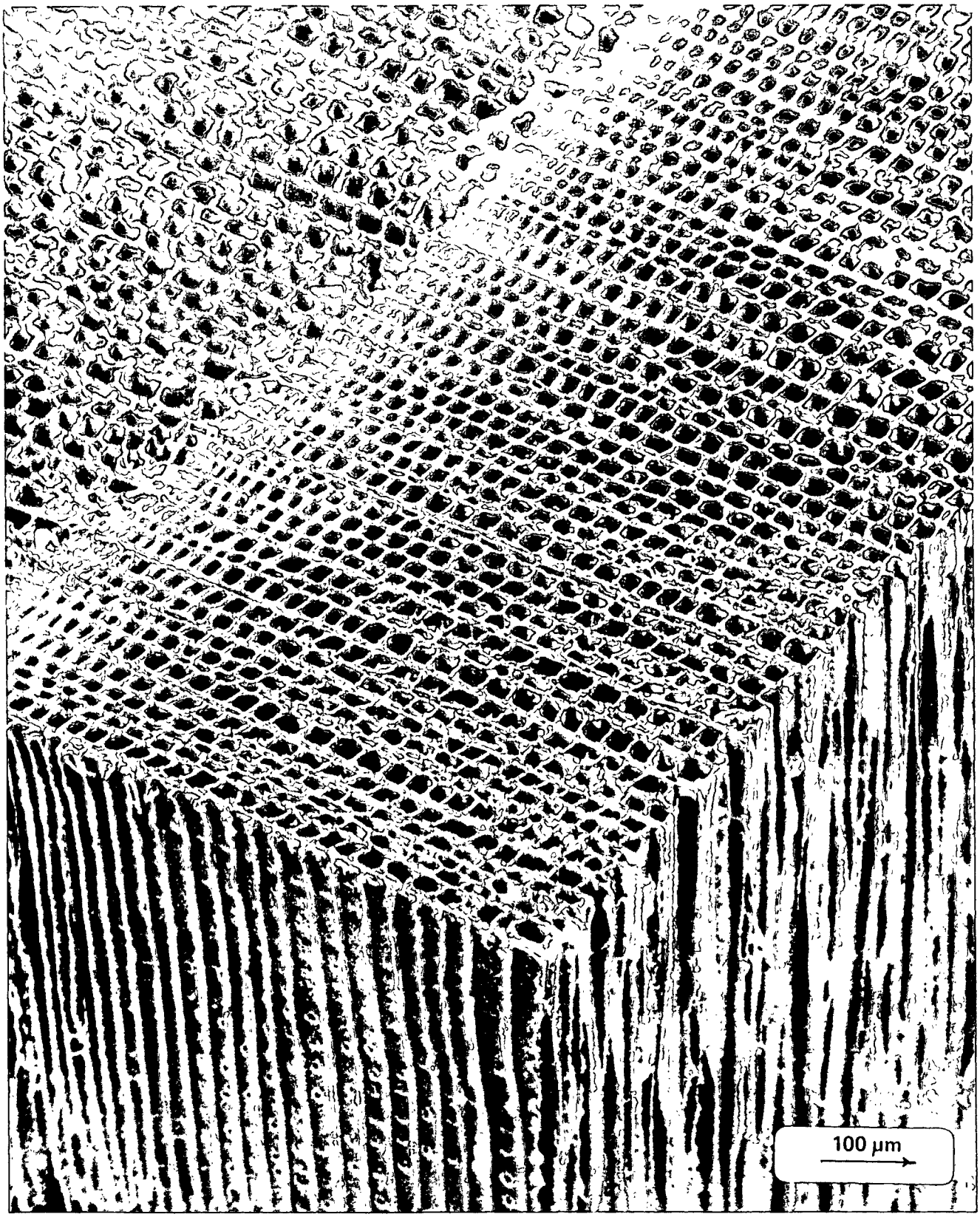


Figure 3. Corner View of a Balsam Fir Wood Block; SEM Plate 1037

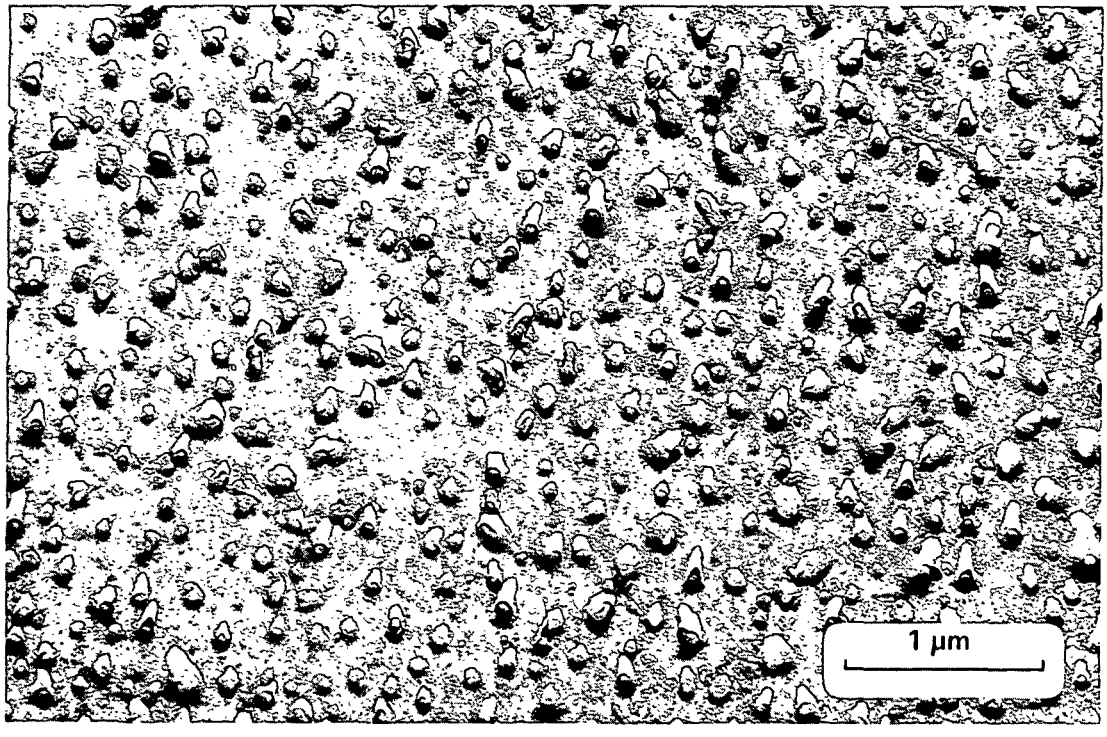


Figure 4. Warty Layer on the Inner Cell Wall Surface of a Mature Balsam Fir Tracheid; Replica, TEM Plate 10245

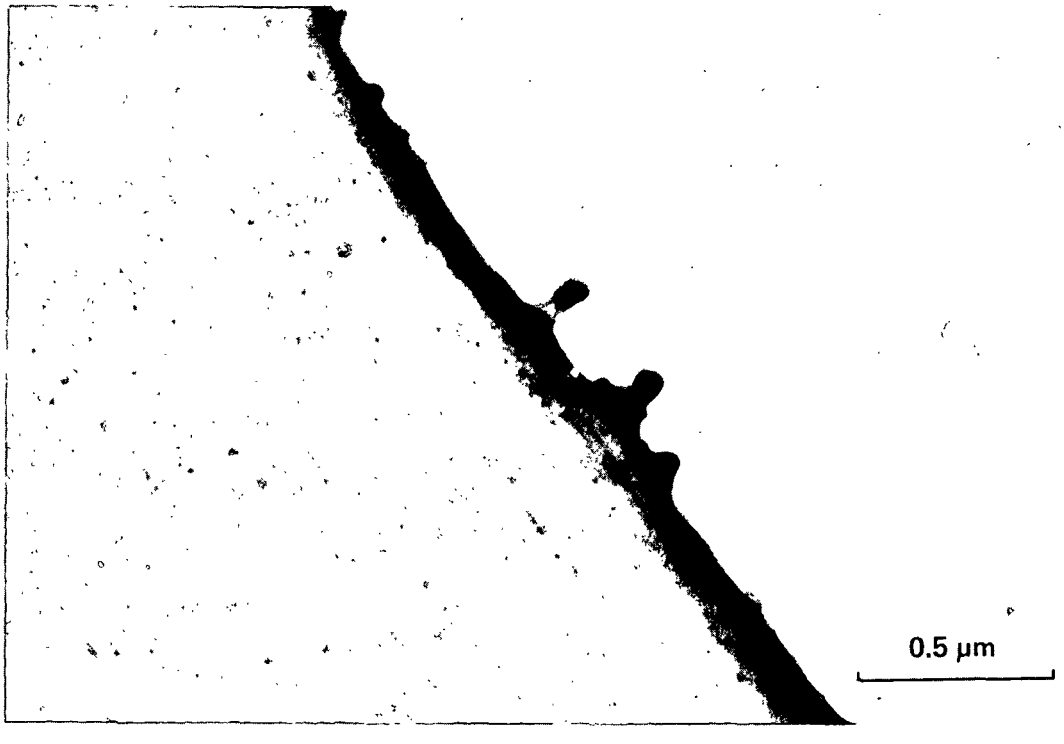


Figure 5. Radial Section of a Mature Balsam Fir Tracheid Wall; $KMnO_4$-Stained UTS, TEM Plate 9170

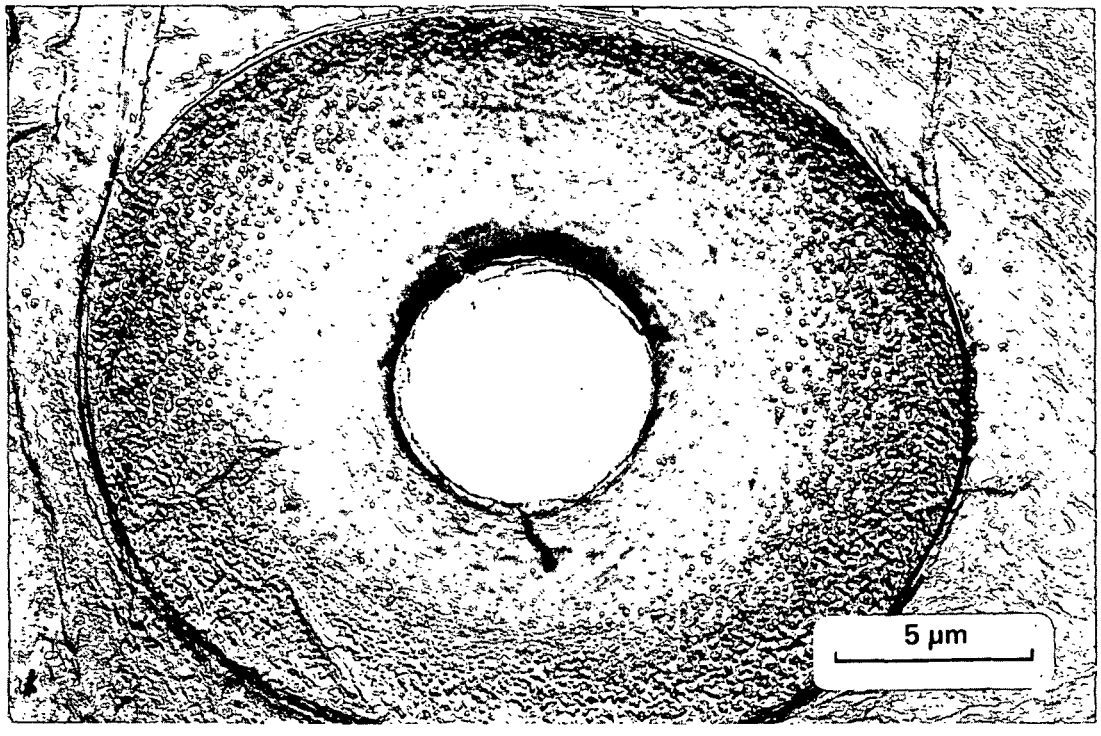


Figure 6. Inner Surface of a Typical Pit Border with Warts Sparse Near the Pit Aperture; Replica, TEM Plate 10258

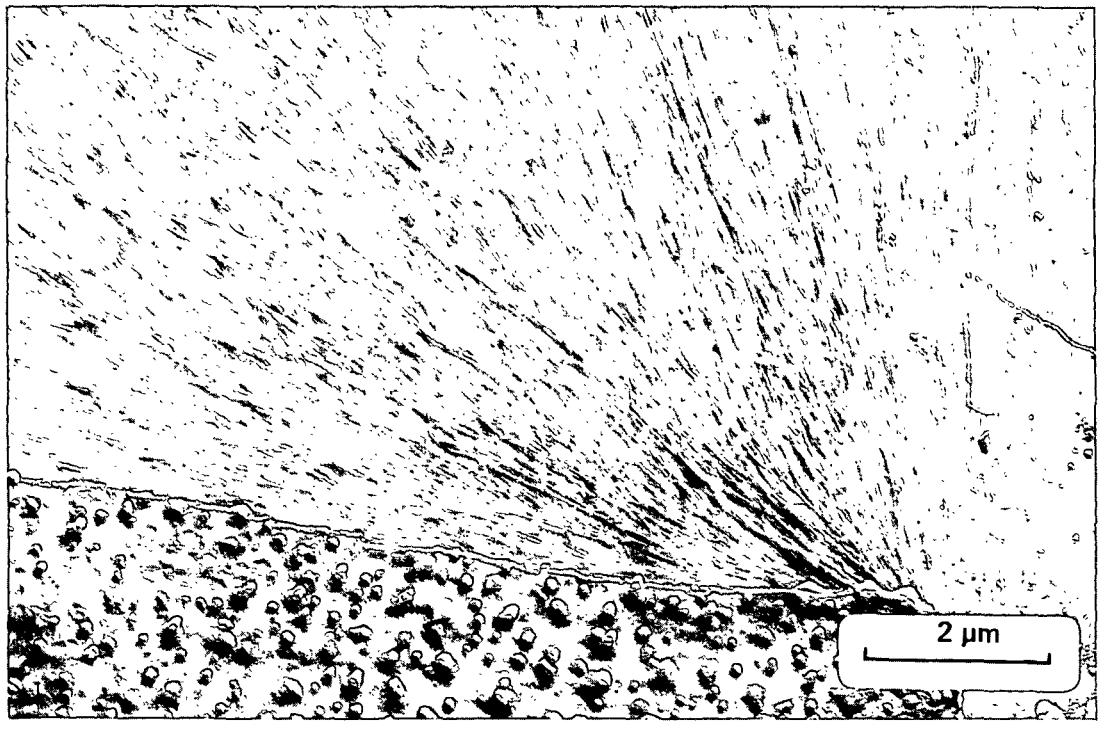


Figure 7. Warty Layer and Exposed Layers of Underlying Secondary Wall; Replica, TEM Plate 10029

The amorphous nature of the warty layer normally obscures the appearance of the underlying secondary cell wall. However, if the wood is sectioned at a slight angle to the radial cell wall, then both the warty layer and the layers of the secondary wall are exposed (Fig. 7). The microfibrillar orientation of the S3 layer, immediately beneath the warty layer, is nearly perpendicular to the fiber axis. Deeper into the cell wall the orientation makes increasingly smaller angles with the fiber axis, and the transition from S3 to S2 is quite gradual. This wall structure has been described by Harada (92) and by Berenzon and Bogomolov (47) for other softwoods.

While the microfibrils in the S2/S3 transition region appear as a spread fan, it is not likely that there is a single lamella having a convergent pattern. Rather, it is more plausible that this region consists of many overlapping lamellae, each with an orientation slightly different from the next (55).

DEVELOPMENT OF THE WARTY LAYER

The study of active cambial tissue can be quite valuable because the sample provides an opportunity to observe wood cells at all stages of differentiation. By examining a single radial file of cells from the cambial initial into the mature xylem, one can observe the growth sequence a single cell would undergo during maturation.

ULTRATHIN SECTIONS OF THE DEVELOPING CELL WALL

Cambial tissue was fixed chemically in an attempt to preserve the cytoplasm as it existed in the living cells at the time of sampling. Such preservation in the differentiating xylem was not always good. According to Robards and Kidwai (93), it is not possible to obtain simultaneously perfect fixation in all cells over the cambial zone because of intercellular variation in osmotic

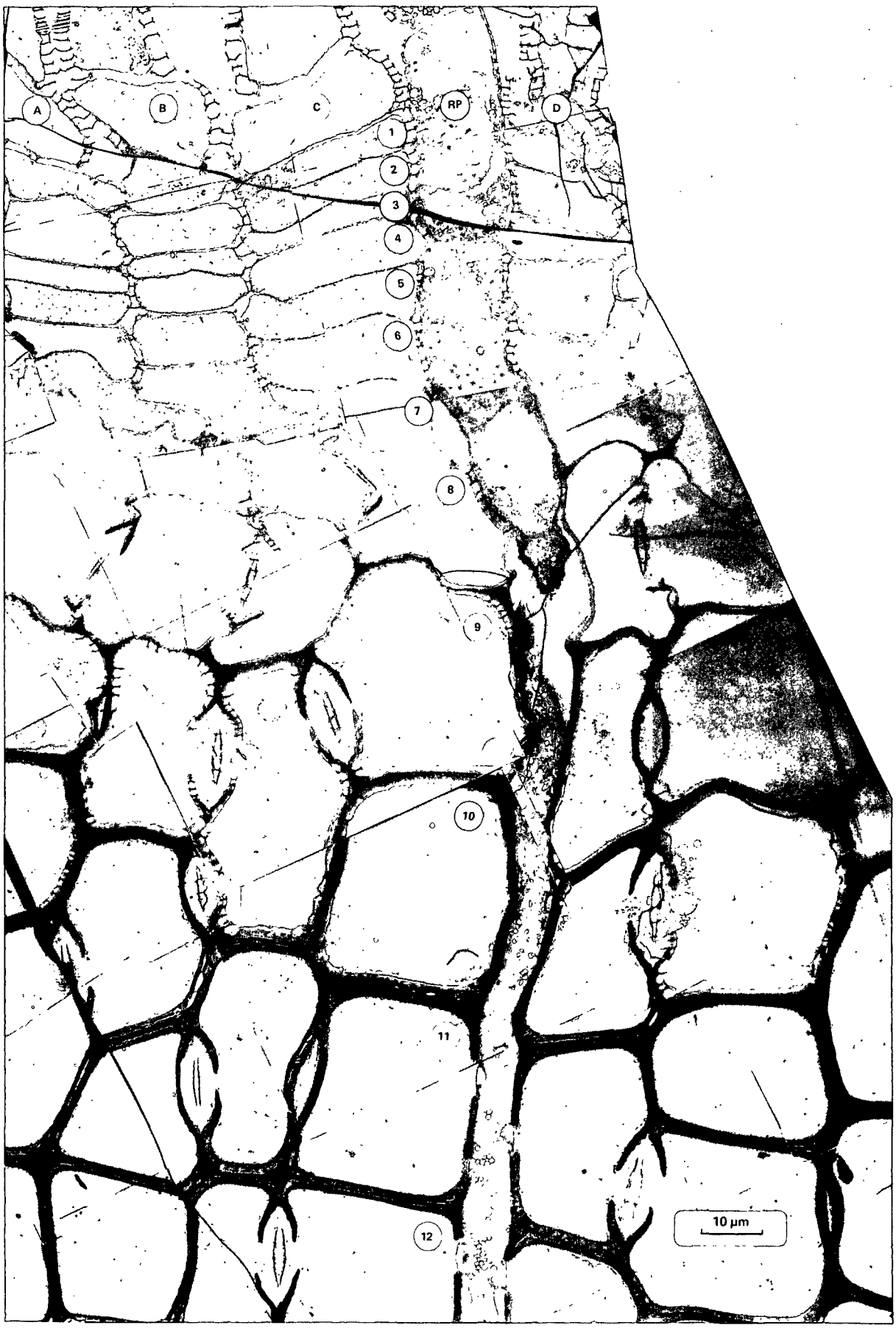
potential, which will result in partial plasmolysis of at least some cells. While this explanation may be more of a rationalization than a fact, it is supported by the results of the present study.

Of the fixatives used in this investigation, potassium permanganate proved best for preservation of cytoplasmic membranes. The permanganate was also the easiest fixative to prepare and use. The aldehyde fixatives were better then permanganate for showing distinctness of the different cell wall layers and for preserving the structure of the developing pit membrane. The Spurr formulation (136) provided the best embedment because its lower viscosity permitted faster penetration into the wood tissue.

As a stain for cut sections, potassium permanganate was very effective in increasing the electron density of lignified tissue. Staining with lead citrate before permanganate enhanced the overall contrast, especially in the cytoplasm, but did not change the pattern of cell wall staining from that of permanganate alone.

Figure 8 is a composite micrograph of the cambial zone and differentiating cells of balsam fir during earlywood formation. The radial files of developing tracheids are designated with Letters A-D. The cells of File C are numbered sequentially from the fusiform initial toward the mature wood. Ray parenchyma cells (RP) are also indicated.

Figure 8. Composite Micrograph of the Cambial Zone and Differentiating Cells of Balsam Fir During Earlywood Formation; Radial Files are Designated A-D; the Developing Tracheids in File C are Numbered Sequentially Toward the Mature Wood, Where 1 is the Cambial Initial; Ray Parenchyma Cells (RP) are Also Indicated; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plates 9791-9795 and 9806-9819


A
B
C
RP
D
1
2
3
4
5
6
7
8
9
10
11
12
10 µm

Developing earlywood provides more desirable experimental material than latewood because cells are produced faster, and the differentiating zone, therefore, includes more cells. The cambial zone in Fig. 8 can be seen as a band of radially-narrow, thin-walled cells. Toward the top of the composite is maturing phloem and toward the bottom is maturing xylem. It is difficult to absolutely identify the fusiform initials in Fig. 8; however, based on discussions by Mahmood (94) and Murmanis (95), reasonable assignments have been made. The designated initials, those cells in tangential alignment with Cell 1 of File C, have thicker tangential walls toward the phloem than toward the xylem as they should since the initial is more active toward the xylem and a complete new primary wall is deposited after each successive division (94). The ray initial is undoubtedly the small ray cell in line with the fusiform initials. Preservation of cytoplasm and organelles in ray cells was generally superior to that in developing tracheids (Fig. 9), probably because the rays are not as highly vacuolated (96).

Wart formation does not occur until the final stages of cell differentiation. However, for a better understanding of the entire process, it is important to follow the development of the cell wall from the initial division to cell death when the living contents degrade and disappear.

Division from the cambial initial and subsequent subdivision of the newly formed mother and daughter cells produces a radial file of new xylem cells each of which is enclosed by primary walls and adjoined by an amorphous intercellular layer, the true middle lamella. Figure 10 shows a wall formed by a recent division in the cambial zone. The new wall indicated by arrow is much thinner than those on either side.

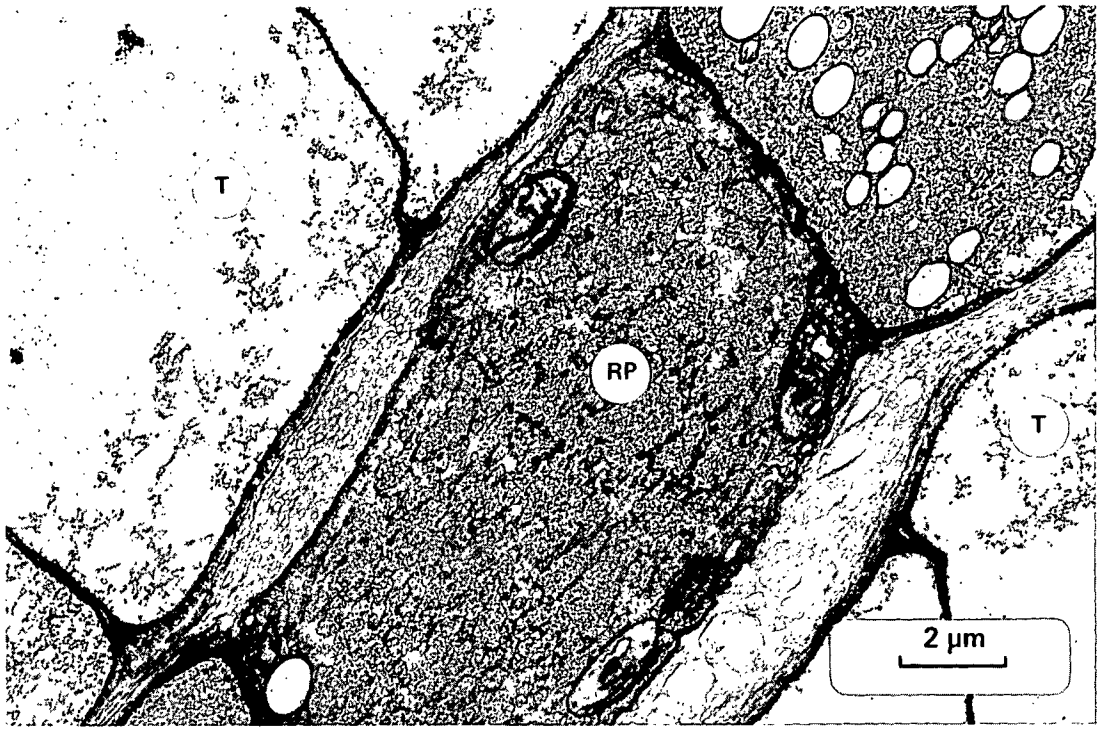


Figure 9. Newly-Formed Longitudinal Tracheids (T) and Ray Parenchyma (RP) in Cambial Zone; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10006

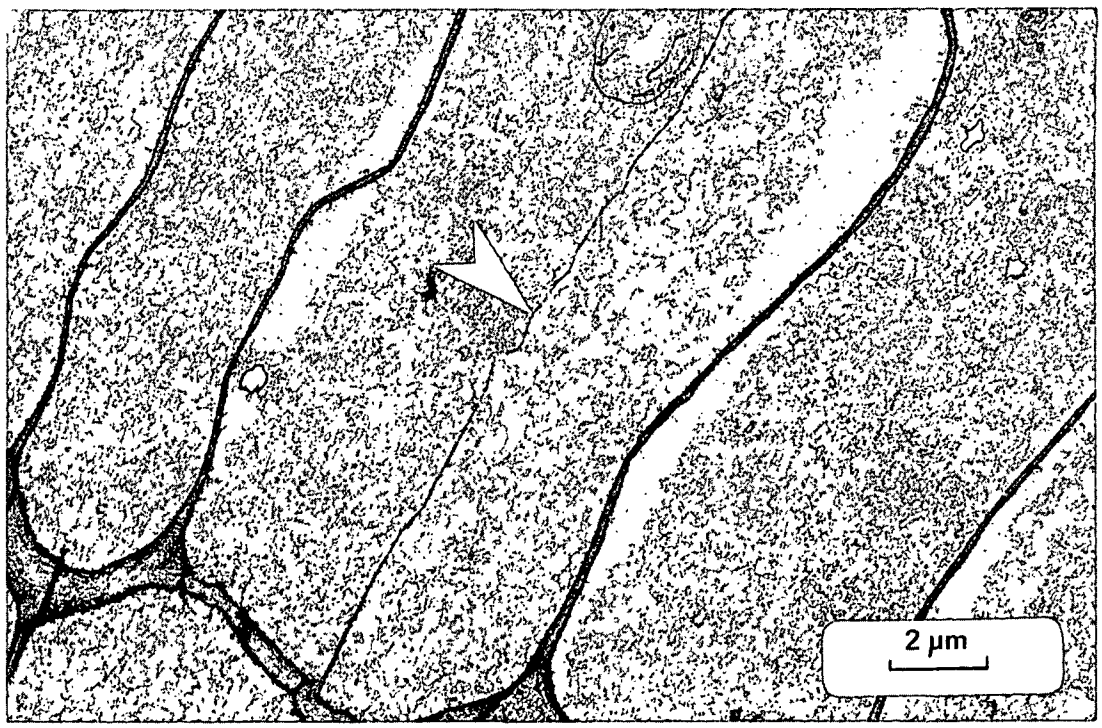


Figure 10. Wall Formed (Arrow) by Recent Cell Division in the Cambial Zone; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10053

After a final division, cell enlargement begins during which the cells increase in their radial dimensions and the secondary wall is deposited. The latter process involves the synthesis and incorporation of polysaccharides into the cell wall. According to Northcote (73), pectins and hemicelluloses are synthesized within the Golgi bodies and transported as membrane-bound material within associated vesicles across the plasmalemma by reverse pinocytosis where they are packed into the wall. Cellulose microfibrils, on the other hand, are probably deposited onto the wall by an enzymatic complex at the external surface of the plasmalemma.

Secondary wall deposition is occurring in Cells 7-11 of File C in Fig. 8. Figure 11 shows the concentration of organelles along a cell wall as secondary deposition nears completion. Golgi bodies, mitochondria, endoplasmic reticulum profiles, and associated vesicles can be observed along the developing tangential wall, between the two cytoplasmic membranes, the plasmalemma and the vacuole tonoplast. Organelles may also be seen in a developing bordered pit of the adjacent cell. [Identification of the organelles in Fig. 11 was based on a discussion of plant cell contents by Clowes and Juniper (97).] The delamination in the cell corner is an artifact that sometimes occurs in sectioning unlignified cell walls.

Bordered pit development, which also occurs during secondary wall deposition, can be observed in the differentiating cells in Fig. 8, especially in Files B and D. The pit borders in contiguous cells do not necessarily develop symmetrically as illustrated in Cell 8 of File C. Figure 12 is a high magnification portion of Fig. 8 depicting a nearly developed bordered pit before warts are formed. Note that the pit membrane is a double primary wall structure which is separated in this figure by swelling in the intercellular region.

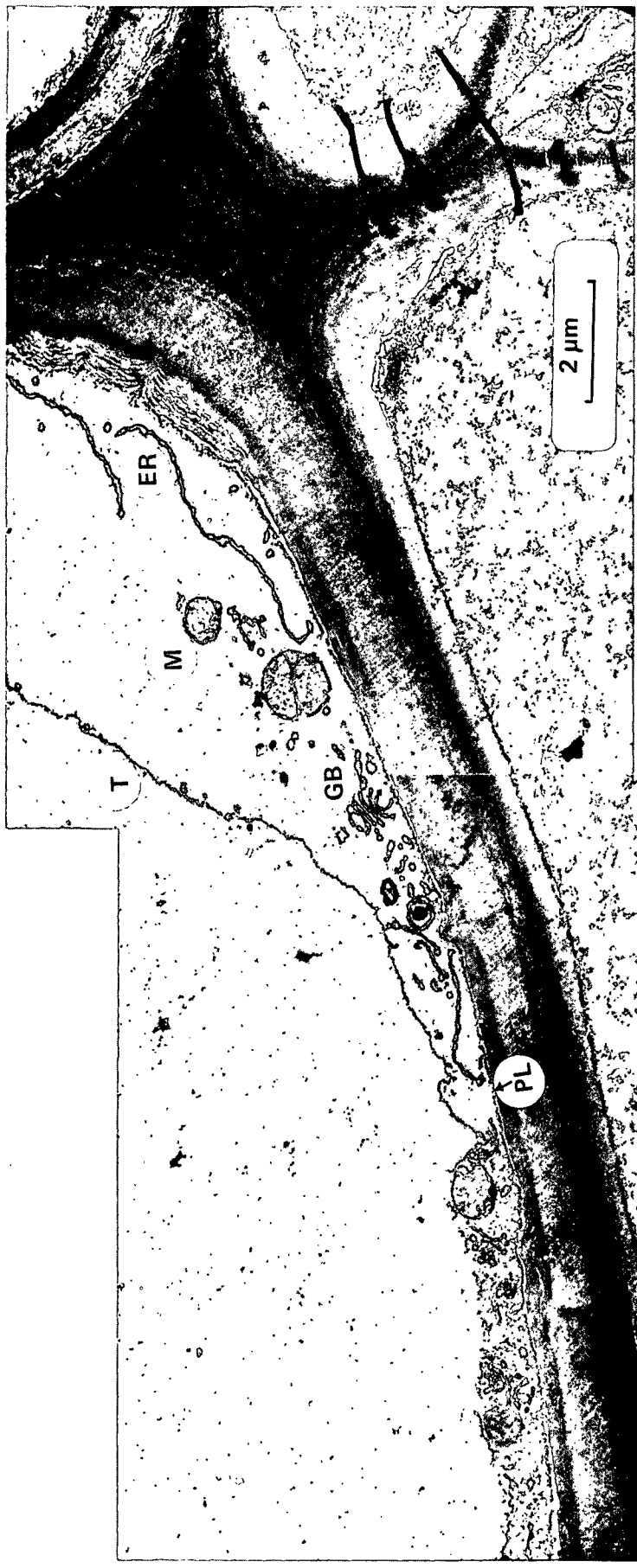


Figure 11. Cytoplasmic Organelles and Membranes Along Developing Tangential Wall: PL = Plasmalemma, GB = Golgi Body, M = Mitochondrion, ER = Endoplasmic Reticulum, T = Tonoplast; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plates 10078 and 10079

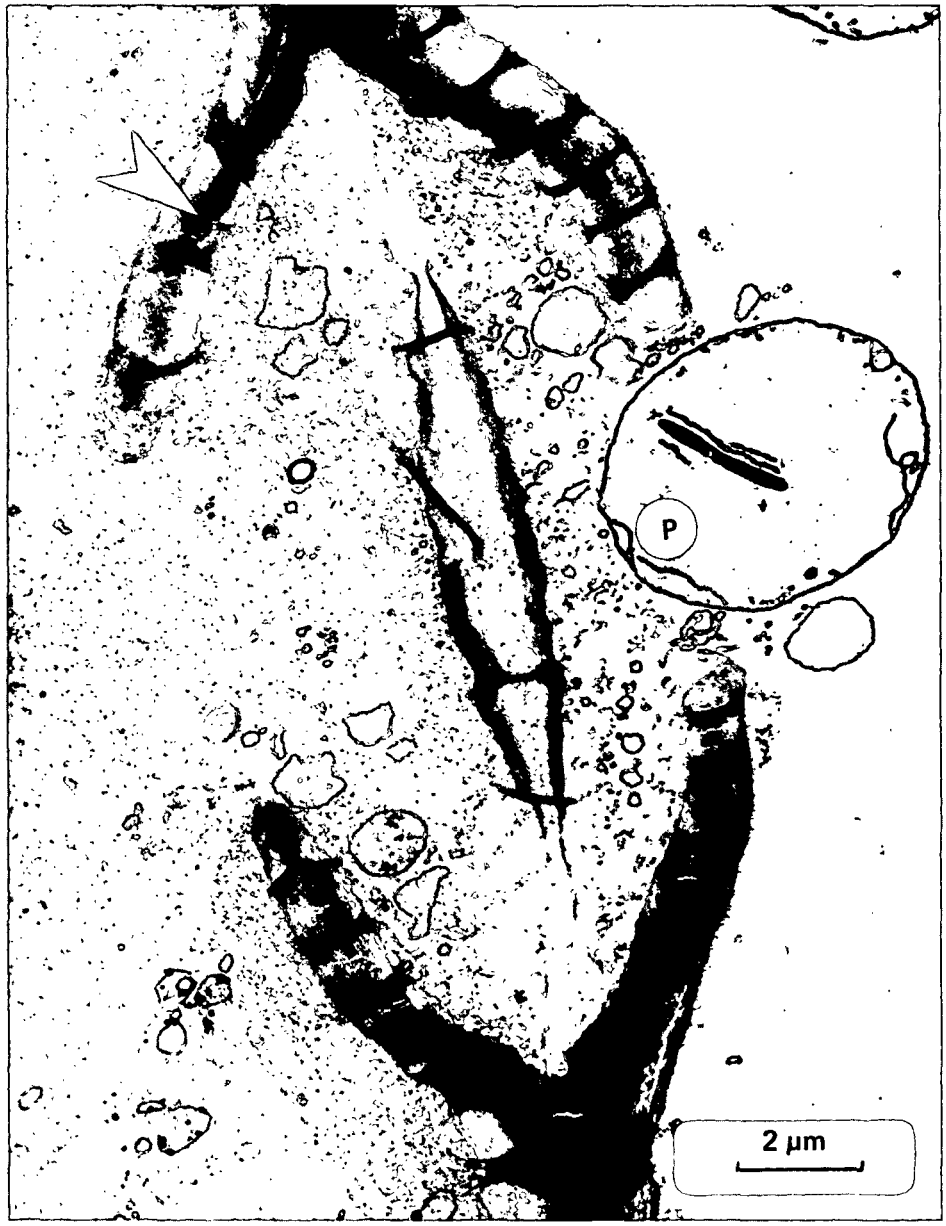


Figure 12. Nearly-Developed Bordered Pit with the Lignification Pattern Evident Within the Initial Pit Borders (Arrow); P = Plastid; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 9820

The large organelle is probably a plastid. The arrow in Fig. 12 indicates the permanganate-stained lignin pattern extending into the initial pit border. According to Schwarzmann (98), lignification proceeds within the initial pit border before other areas of the secondary cell wall.

The major part of wood lignification occurs after secondary wall deposition is complete and before wart formation. Lignin can be seen in ultrathin wood sections because of its staining reaction with potassium permanganate, which has been shown to be specific for the lignin component of the cell wall (99, 103). The darkly stained walls of the last two or three maturing cells of each file in Fig. 8 indicate completed lignification. In the next few cells nearer the cambium, lignification had begun but was not complete.

After the intercellular region was lignified, wall lignification began in the cell corners just inside the primary wall and proceeded through the walls of a cell beginning in the S1 and continuing toward the lumen. Figure 13 shows the wall between Cells 9 and 10 of File C in Fig. 8. In the initial stages of lignification the S1 appears more darkly stained than the primary wall or the rest of the secondary wall (see arrow in Fig. 13). This has also been shown in developing balsam fir tracheids by Schwarzmann (98). He reported that lignification first extends along the tangential wall farthest from the cambium, then proceeds along the radial walls and finally along the tangential wall nearest to the cambium. Wardrop (100), however, reported that both tangential walls appear to become lignified somewhat before the adjacent radial walls. In the present study neither described pattern of wall lignification was consistently observed. As could best be determined, lignification began in the corners but then proceeded in all four walls of a cell nearly simultaneously.

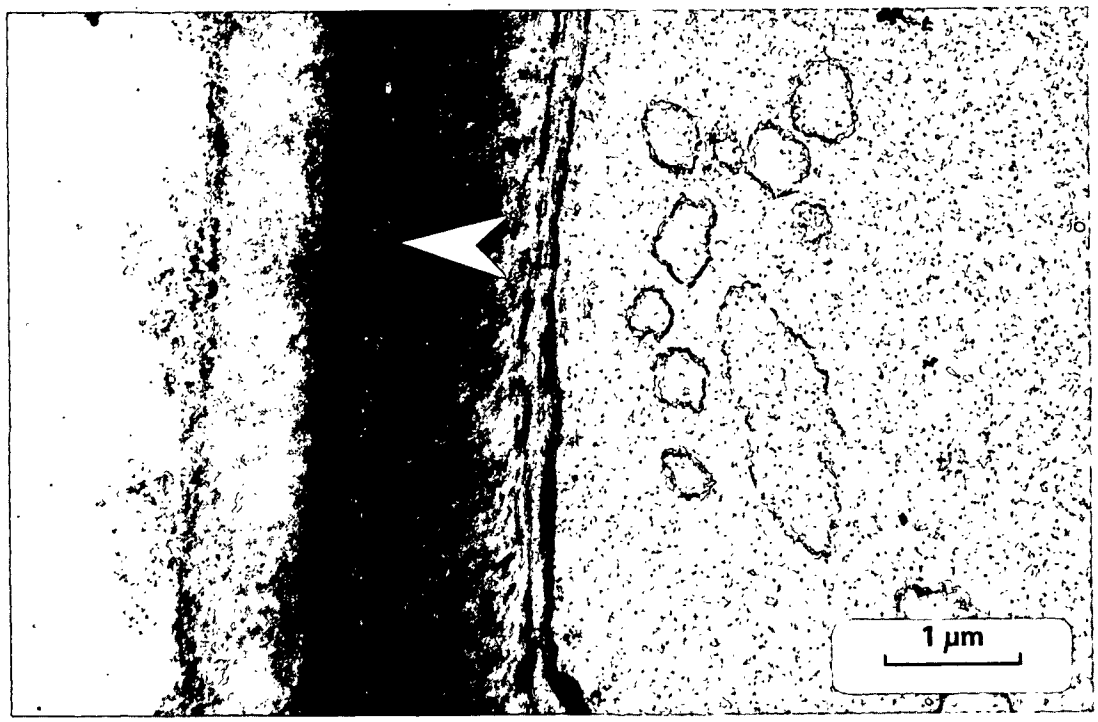


Figure 13. Early Stage of Lignification in Tracheid Tangential Wall; Intercellular Region and S1/S2 Interface (Arrow) Appear More Darkly Stained than the Rest of the Wall; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 9800

Figure 14. Double Tangential Wall Between Adjacent Tracheids Which are in Different Stages of Lignification; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10247

Two conflicting theories concerning the origin of lignin precursors are: (1) they are formed in the cambial zone and are subsequently transported into the matrix of secondarily thickened walls where polymerization takes place, e.g., (101), and (2) they are synthesized within the individual differentiating cells and then incorporated into the cell walls, e.g., (102). Figure 14 shows the double tangential wall between adjacent cells in radial File A of Fig. 8. The wall farther from the cambium is more completely lignified as shown by the darker permanganate staining. Since both polymeric lignin and its monomeric precursors would presumably be stained with permanganate (103), this micrograph is evidence that the introduction of precursors to the cell wall is directed by the cell. If the introduction of lignin precursors were directed from outside the cell, one would expect lignification to proceed equally in both halves of a double cell wall. This evidence does not exclude the possibility that one cell wall by some intracellular process is made more receptive than another to lignin precursors produced and transported from elsewhere. However, the most plausible explanation in light of the existing data is that precursors and any lignification-controlling functions originate within the individual cell and that the lignification process itself begins and ends entirely under individual cell direction. According to this explanation, the precursors, in moving from the cytoplasm to the lignification site are too dilute to give a dark staining reaction.

No organelle was observed to be directly associated with lignin synthesis or incorporation since none were characteristically present near the areas of lignin deposition. Hepler, et al. (99) also could find no organelle or discernible cytoplasmic structure involved in the process of lignification. Pickett-Heaps (104), however, contended that Golgi bodies and associated vesicles may add tritiated lignin precursors to the wall.

Warts first develop at about the time cell wall lignification is complete. Schwarzmann (98) has shown evidence that wart formation may occur before the entire S3 layer is lignified; however, in the present study, wart formation was observed only after S3 lignification was complete. In File C of Fig. 8, warts first occur in Cell 11. Analogous to the lignin depositional pattern, warts develop first in cell corners and then on the radial and tangential walls nearly simultaneously. Warts form in pit cavities at about the same time that they are forming in cell corners of the same tracheid. Interestingly, the distribution of warts on the surface of the inner pit border (see Fig. 6) corresponds to the extension of lignified areas within the pit border (see Fig. 12, arrow).

Warts seem to appear first as small mounds that stain slightly darker with permanganate than the rest of the secondary wall (Fig. 15). (It is necessary to examine all the developing warts on the cell wall in cross section to be sure that they are actually small mounds and not tall, mature warts merely sectioned off-center.) Warts continue to protrude into the lumen and, when mature, they, along with the amorphous terminal layer, stain much more darkly than the rest of the cell wall (see Fig. 5). Wart formation in developing latewood tracheids follows the same course as in earlywood.

An important observation is that warts are developed exterior to the plasmalemma before the cytoplasm degenerates and is lost from the cell. Figures 16 and 17 show recently developed warty layers in living cells of earlywood and latewood, respectively. It appears that some cytoplasmic contents have begun to deteriorate, perhaps due to incomplete fixation, but it is clear that warts are definitely formed while the cytoplasm is still present. The warty layer, therefore, cannot consist of desiccated cytoplasmic membranes with organelles trapped between to form the protrusions. This conclusion is consistent with earlier studies (19, 65).

Figure 15. Beginning of Wart Formation on the Cell Wall Exterior to the Plasmalemma; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10086

No organelle was found to be associated with wart formation, although occasionally the plasmalemma appeared to retract slightly from the wart structure, sometimes breaking, and giving the appearance of some vesicular material (Fig. 15 and 18). This occurrence may have been a result of partial plasmolysis in the imperfectly fixed cell or it may represent plasmalemma invagination as described by Timell (96). There was no unique feature observed within the cell wall beneath the forming wart.

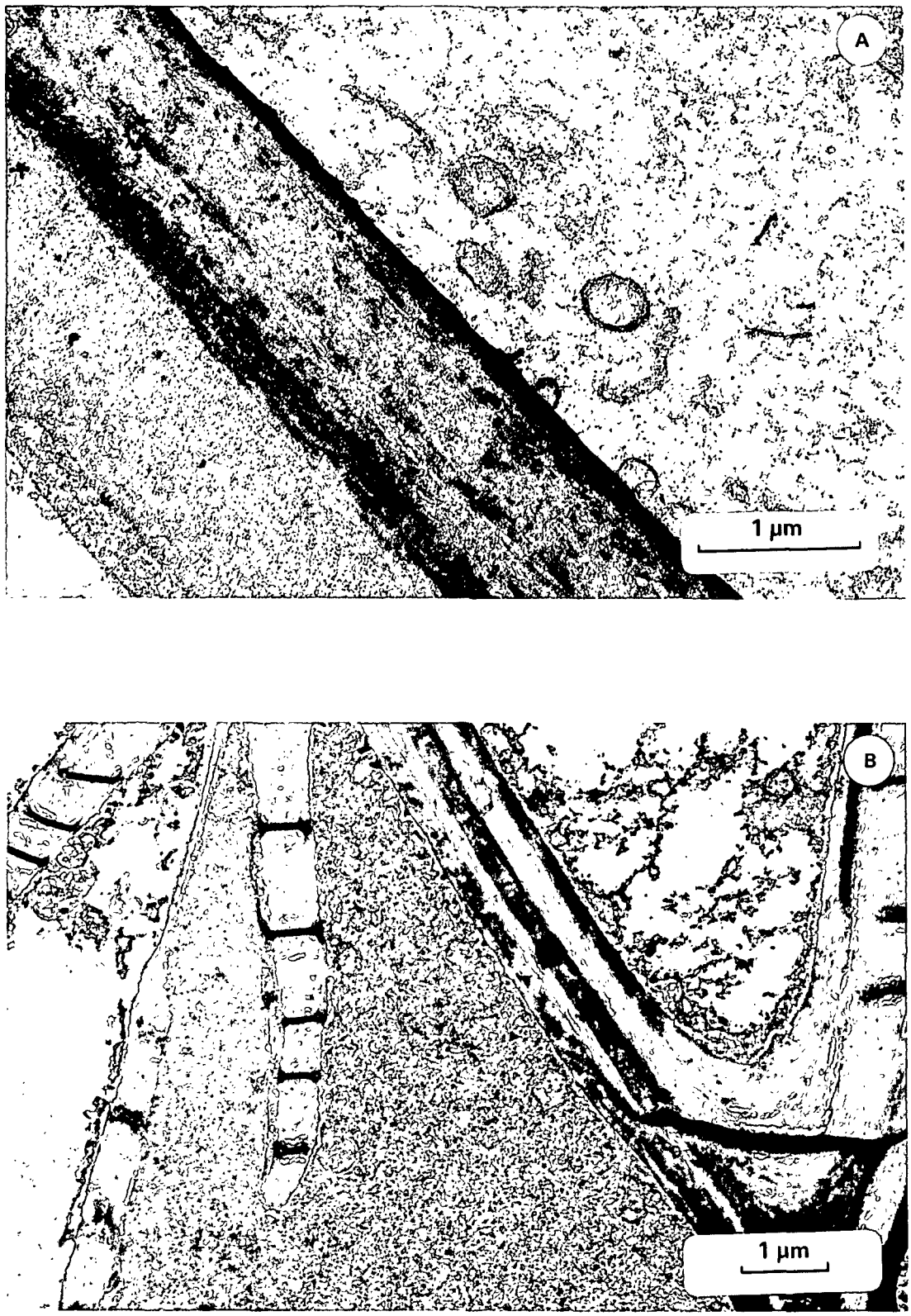


Figure 16A and 16B. Recently Developed Mature Warty Layers in Living Earlywood Tracheids with Cytoplasm Still Present; 16A. June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 9805; 16B. June 12 Sample, FAG-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 9770

Figure 17. Recently Developed Mature Warty Layer in Living Latewood Tracheid with Cytoplasm Still Present; July 13 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10016

In mature tracheids, warts were more numerous and larger in the corners than along the walls (Fig. 19). In a few cases, an interior, basal portion of the warts along the cell wall stained less darkly with permanganate than the rest of the wart structure (Fig. 20). This may indicate a two-component composition for the warty layer.

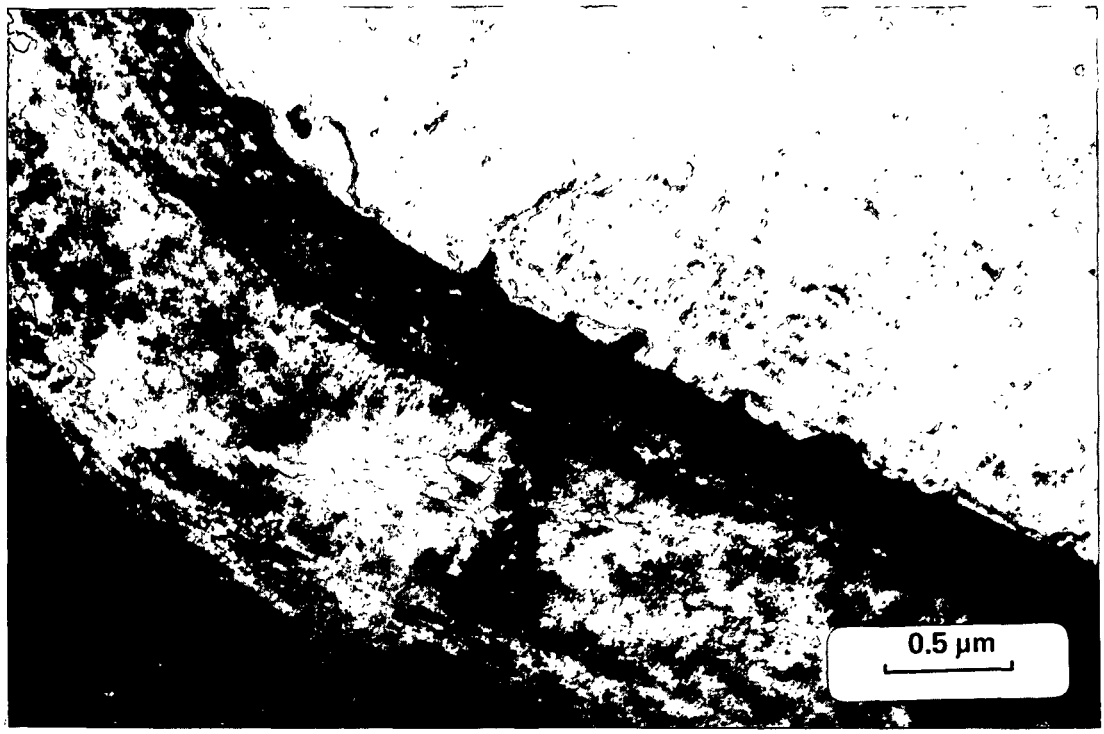


Figure 18. Newly-Formed Warty Layer with Plasmalemma Retracted and Broken in a Few Places; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 9781

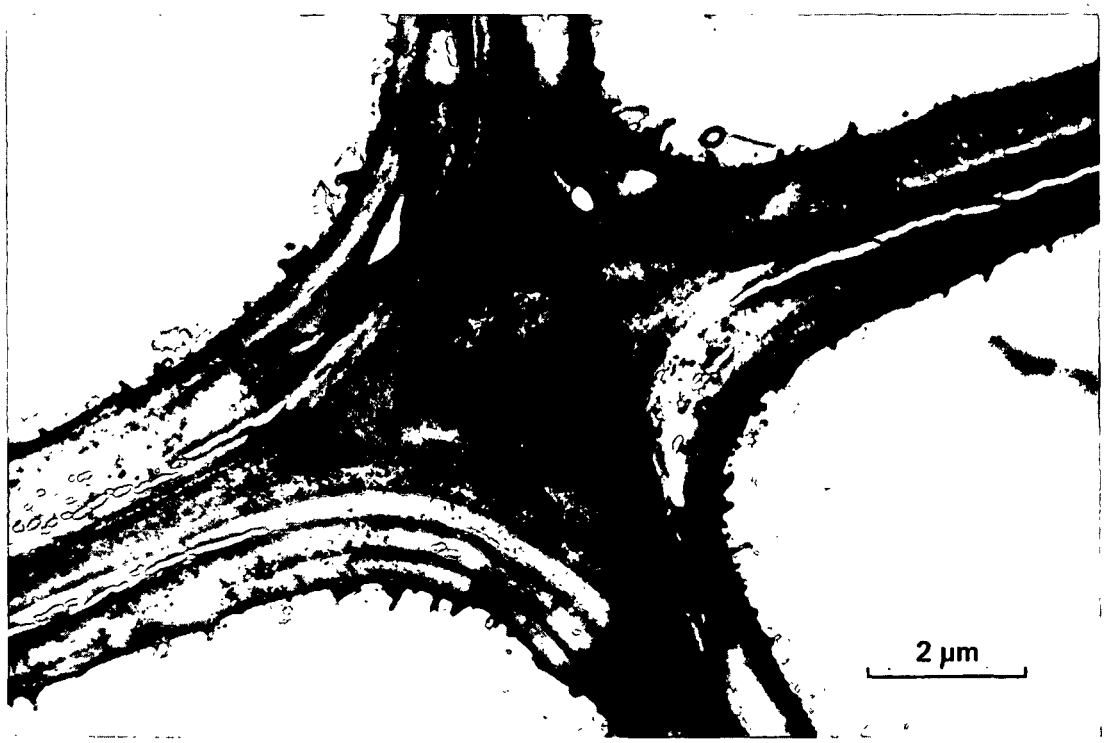


Figure 19. Micrograph Illustrating the Typical Situation Where Warts are More Numerous and Larger in the Cell Corners than Along the Walls; June 12 Sample, $KMnO_4$-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10246

Figure 20. Mature Cell Wall with Interior of Some Warts not as Darkly Stained as Outer Portion; July 13 Sample, GAG-Fixed, Pb Citrate-and $KMnO_4$-Stained UTS, TEM Plate 10056

Wart formation was the last step in cell wall development. Shortly after the warty layer was elaborated, the cytoplasm disappeared from the cell without leaving any apparent, disorganized residue.

SURFACE EXAMINATION OF THE DEVELOPING CELL WALL

Additional evidence on the development of the cell wall in differentiating balsam fir tracheids came from examination of surface replicas of cambial tissue prepared by sectioning unfixed, collodion-embedded samples. Developing earlywood provides the best experimental material for examining the inner surface of differentiating cells since earlywood cells have a larger radial dimension than latewood cells. Figures 21A-21H show the inner surface of tracheids as they develop in a radial file moving inward from the cambium. The wall exposed in each cell is the last portion of the wall deposited when the sample was taken. Figures 21A and 21B are of two adjacent tracheids, and the rest of the series are of six adjacent tracheids from a different file. (Tracheid axis is parallel with the long axis of each micrograph in Fig. 21.) The entire set corresponds to Cells 5 or 6 through 12 in File C of Fig. 8. The series is representative of the sequential cell wall layers deposited on a single tracheid as it develops. (Refer to Fig. 1 for a model of the layers in a mature softwood cell wall.)

Figure 21A shows the irregular orientation of the microfibrils on the inner surface of the primary wall, which is deposited after the initial division in the cambial zone. Figure 21B illustrates a lamella in the S1 layer with the microfibrils oriented almost normal to the tracheid axis, following a flat helical pattern. The microfibrils in the S2 layer (Fig. 21C) display a steep helical pattern oriented 10°-20° to the fiber axis. Figure 21D shows a more interior part of the S2 layer with microfibrils oriented nearly the same as in the preceding cell. In Fig. 21E is shown the S3 layer with microfibrils oriented at about 70° to the fiber axis, forming a relatively flat helix.

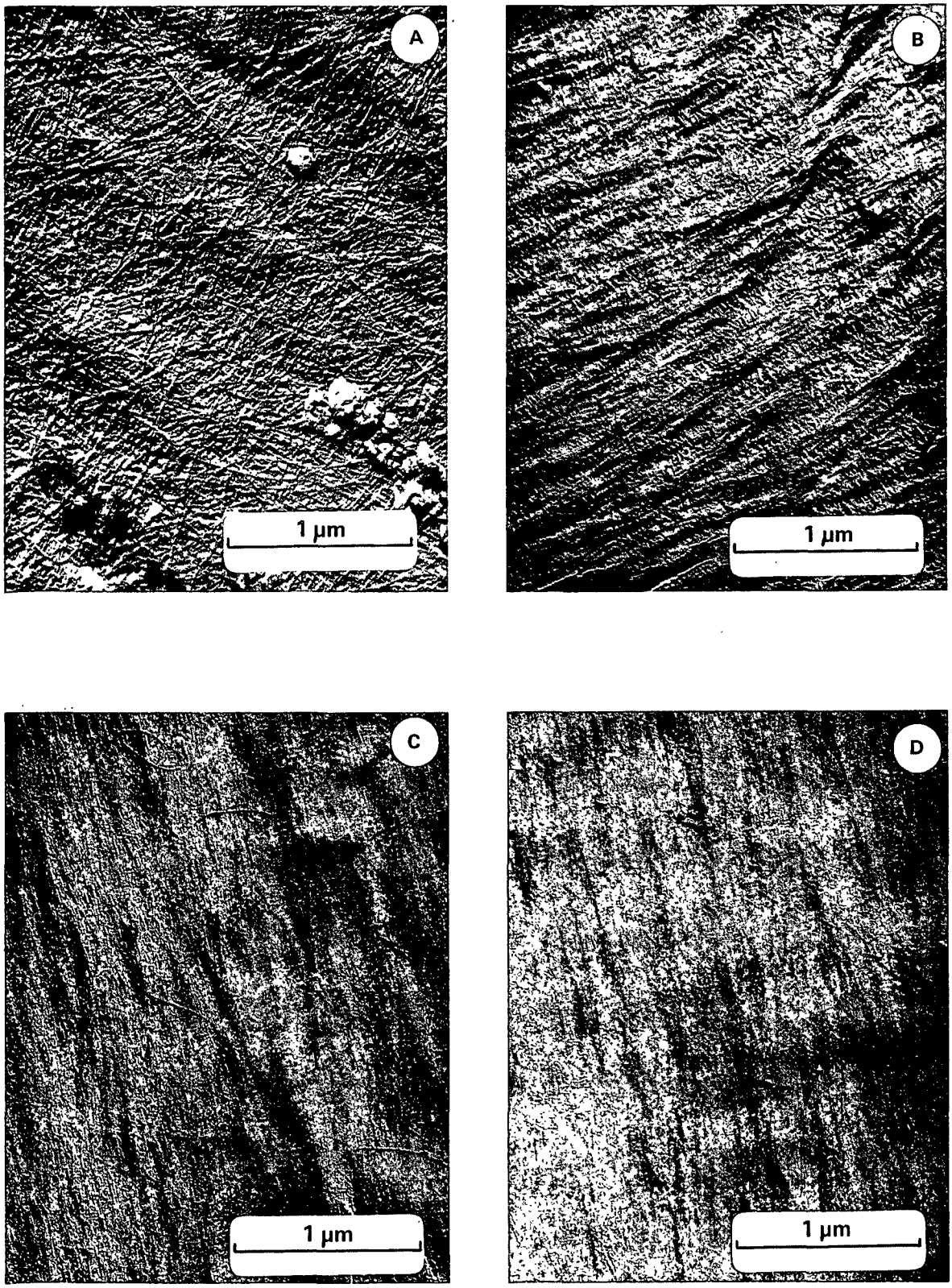


Figure 21A-21D. Inner Surface of Developing Tracheids in a Radial File Moving Inward from the Cambium: A. Primary Wall, B. S1 Layer, C. Exterior Part of S2 Layer, D. Interior Part of S2 Layer; June 12 Sample, Replica, TEM Plates 10018, 10019, 10036, and 10037

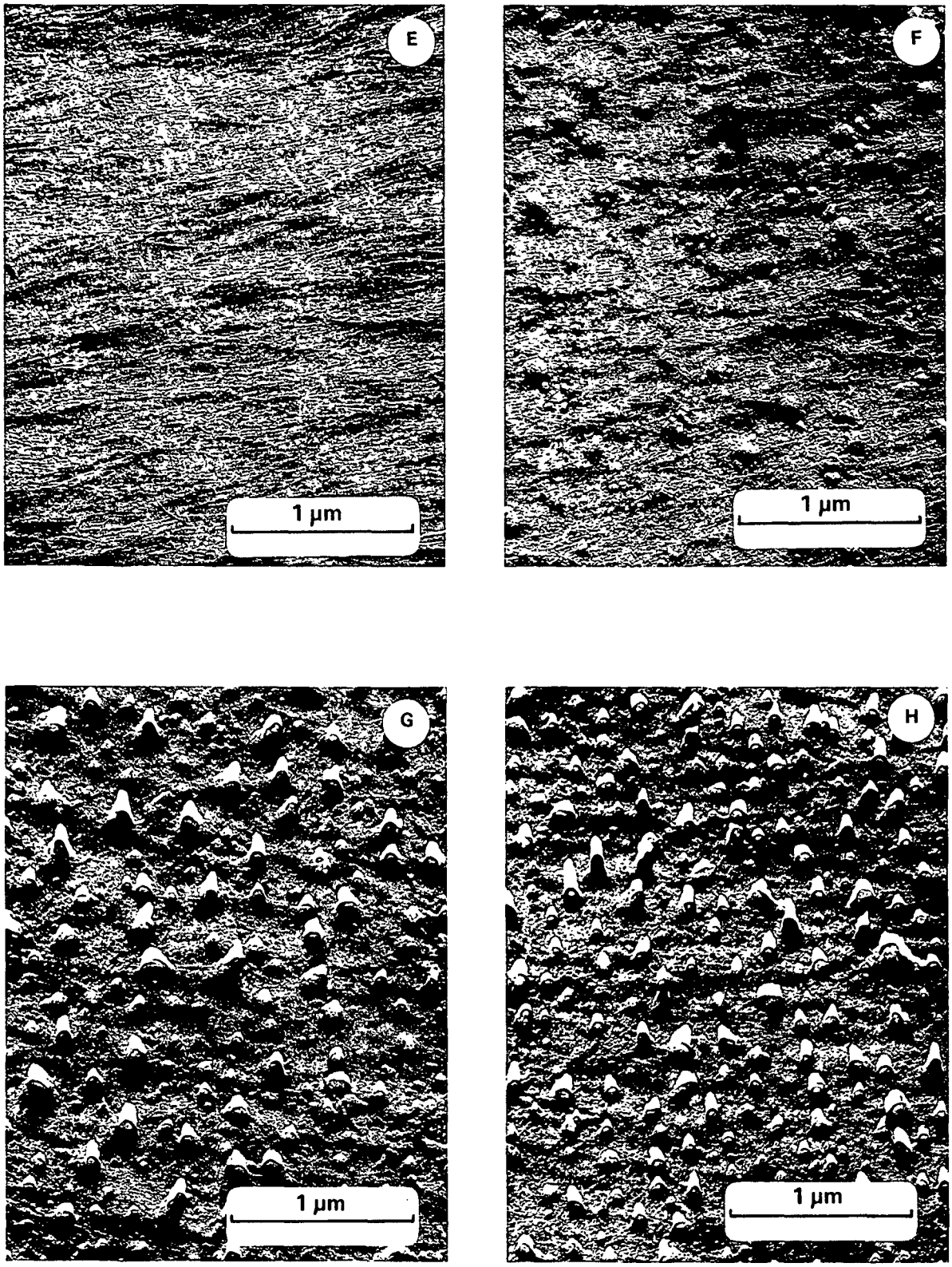


Figure 21E-21H. Inner Surface of Developing Tracheids in a Radial File Moving Inward from the Cambium: E. S3 Layer, F. Immature Warty Layer, G. Nearly-Mature Warty Layer, H. Mature Warty Layer; June 12 Sample, Replica, TEM Plates 10038-10041

In the material examined, no developing cells were observed in which the microfibrillar orientation of the last-deposited lamella was intermediate between the orientation of the S1 and S2 or between that of the S2 and S3. Therefore, any intermediate lamellae are deposited and then covered quite rapidly. Figure 21F exhibits the S3, partially encrusted, but the microfibrillar orientation is still evident. This micrograph is a surface view of the warts in an early stage of formation. Figure 21G shows a nearly mature warty layer. The microfibrillar orientation of the underlying S3 layer is now totally obscured. These two micrographs, Fig. 21F and 21G, confirm the fact that mature warts develop over a period of time and do not appear suddenly as reported by Wardrop, et al. (16). The next cell (Fig. 21H) displays the mature warty layer of a completely differentiated cell. The warts here are more numerous, indicating that a few more are formed in this final interval.

The radial face and corner inside a differentiating cell is shown in Fig. 22. Warts on the radial face appear as low mounds, while those in the corner appear to protrude higher into the lumen. This micrograph illustrates and confirms the fact that warts develop earlier in cell corners.

The electron micrographs, Fig. 21A-21H and 22, were purposely taken of wall surface areas not obscured by plasmolyzed cytoplasm. Figures 23A and 23B show evidence of cytoplasm over developing warts and mature warts, respectively. These micrographs provide further confirmation that the cell is living when the warty layer forms. The desiccated cytoplasmic material often appeared as strands aligned perpendicular to the fiber axis in the more completely developed cells. The orientation was nearly parallel to the orientation of the last-deposited S3 microfibrils. Such observation may indicate a relationship between some organization in the cytoplasm, or its

membranes, and the orientation of the deposited microfibrils. In cells beyond the differentiating zone, there was no evidence of desiccated cytoplasm anywhere on the cell wall. By this time all the cytoplasm and associated organelles and membranes had degraded and left the cell. This is contrary to Wardrop and Harada (105) who contended that the final layer of the mature cell consists of denatured remnants of the cytoplasm.

Figure 22. Radial Face and Corner Displaying Developing Warty Layer; June 12 Sample, Replica, TEM Plate 10043

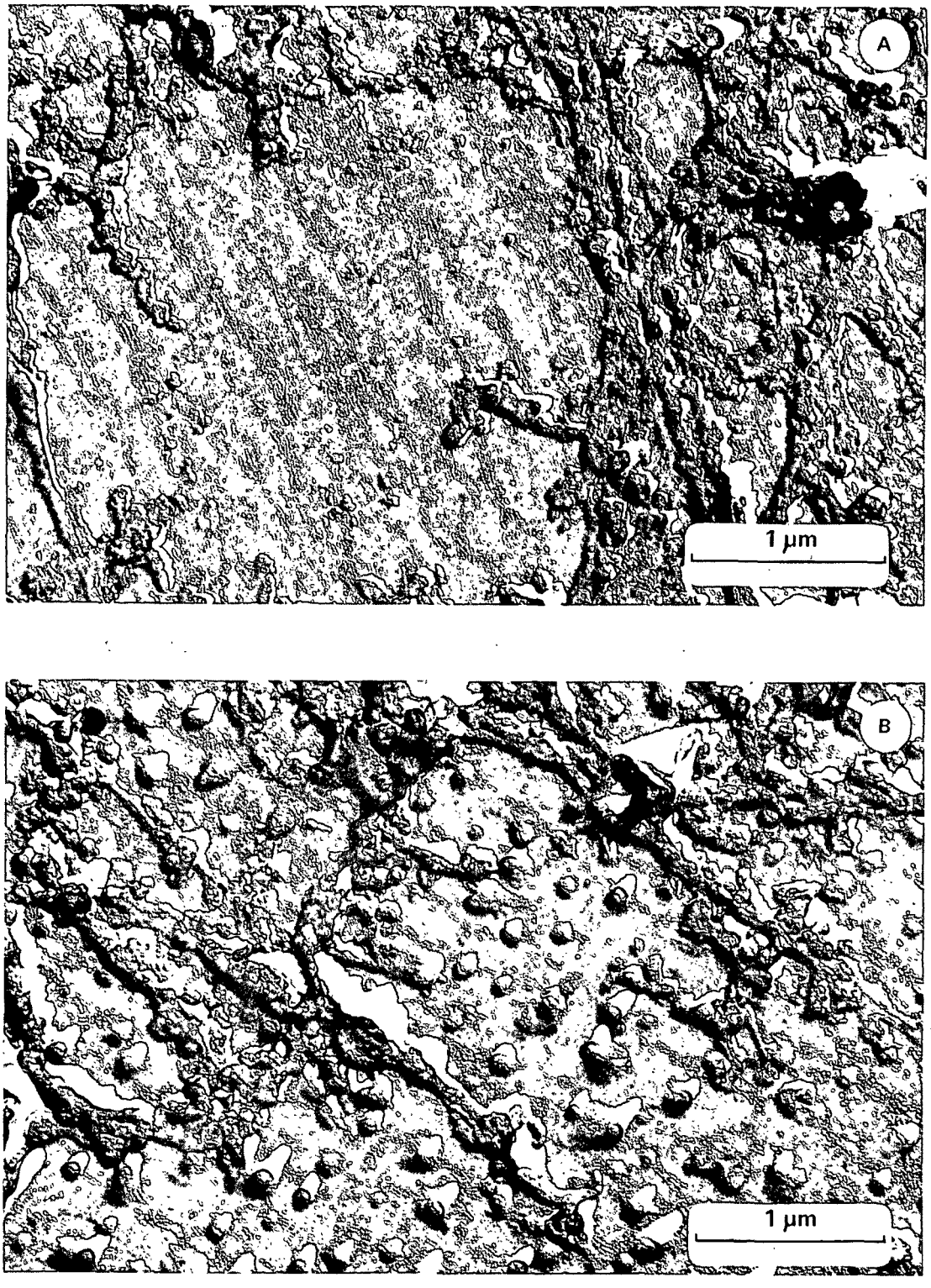


Figure 23A and 23B. Cytoplasmic Remains over a Developing Warty Layer: A. Immature Warts, B. Mature Warts; June 12 Sample, Replica, TEM Plate 10042 and 10048

In the study of the last-deposited cell wall in differentiating tissue, no evidence was found for Wardrop and Harada's conclusion (105) that secondary wall thickening begins near the center of cells and progresses toward the cell tips. In all developing tracheids examined, the same microfibrillar orientation occurred over the whole length of the cell visible. (The sections were often slightly oblique, preventing examination of the entire cell, tip to tip.) The present findings are consistent with Wooding (106) who found that incorporation of both labeled glucose and phenylalanine was simultaneous at all points along the wall.

In conjunction with the examination of the developing cell wall, membranes in differentiating bordered pits were also studied. The sequential steps in the development and perforation of the intertracheid pit membrane is shown in Fig. 24A-24D. Figure 24A shows the primary wall pit structure and the beginning of secondary thickening to form the pit border. Microfibrils in the membrane region are randomly oriented and no torus is evident yet. From ultrathin sections, it is apparent that torus thickening begins shortly after the time that secondary wall deposition and pit border formation begins (see Fig. 8). This is not to say, however, that the torus is similar to secondary wall material. The origin of bumps observed on this early membrane structure (Fig. 24A) is unknown, though they could represent the beginning of torus formation. Figure 24B shows the lumen-side view of a pit with the borders more developed. Here the central torus is evident with circularly oriented microfibrils at the periphery. The outer margo region remains unperforated. Because of membrane encrustation, only the larger, radially apposed microfibrils are easily seen in the developing margo.

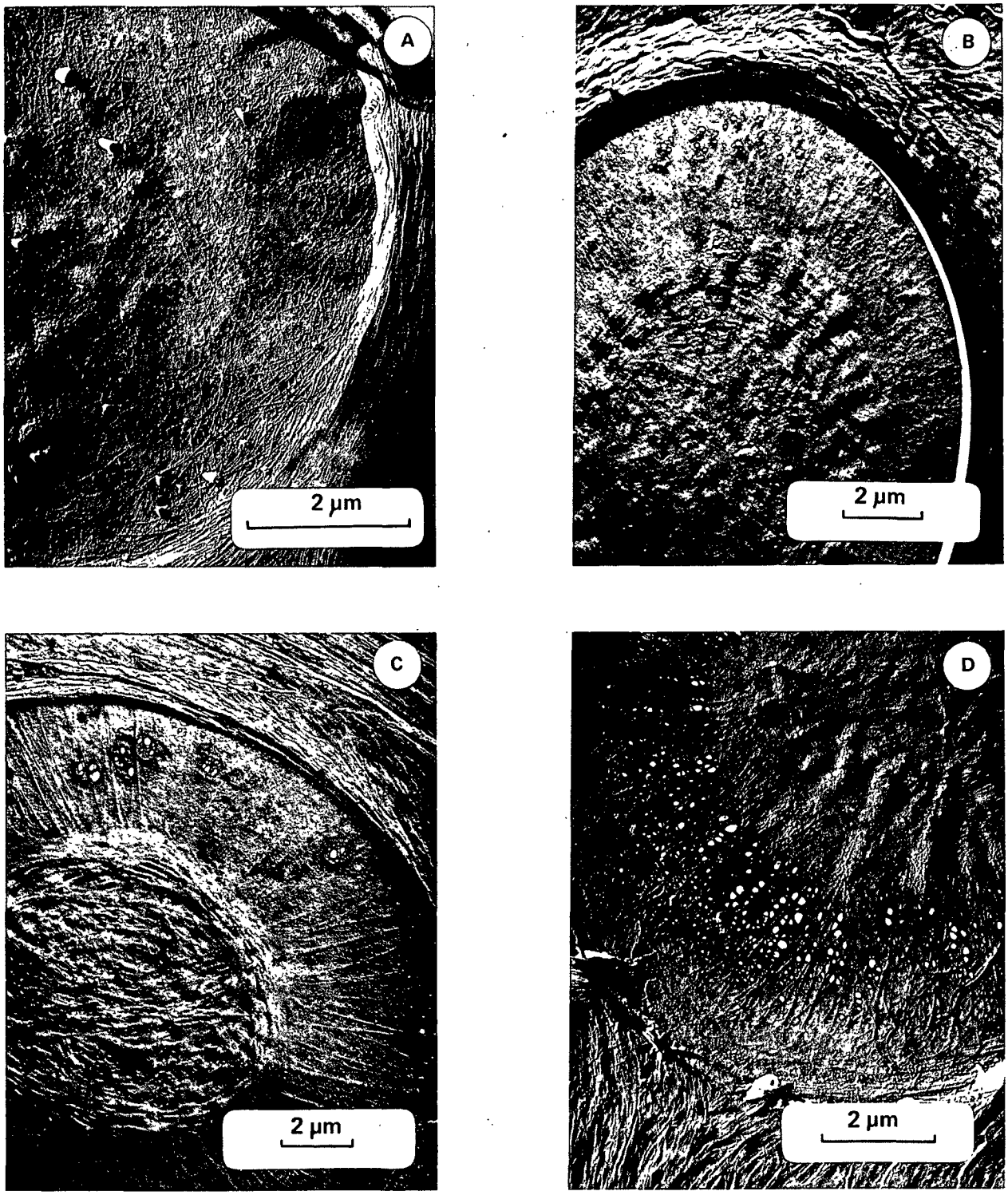


Figure 24A-24D. Stages in the Development and Perforation of the Membrane in Intertracheid Bordered Pits: A. Primary Wall Pit Structure, B. Developed Torus and Imperforate Margo, C. Initial Perforation of Margo, D. Further Perforation of Margo; June 12 Sample, Replicas, TEM Plates 10244, 10139, 10210, and 10145

Figure 24C shows a split wood section and initial perforation of the margo region. This phenomenon does not proceed in membrane development until after wart formation is complete on the cell wall. The perforation process probably begins at the time of overall cell autolysis, shortly before the cytoplasmic material is eluted from the cell. The process may involve a combination effect of enzymatic action of the withdrawing cell contents and later physical erosion by the intercellular transpiration stream (107). Figure 24D shows further perforation in the margo region.

Figure 25 shows a mature pit membrane with perforation essentially complete. The warts are visible through a torn portion of the membrane, on the underlying pit border. Except for the tear this is presumably how the mature pit membrane appears *in vivo*. However, drying and preparation procedures for electron microscopy may have caused undetermined structural changes.

Additional illustration of membrane perforation was provided by ultrathin sections of differentiating tracheids. Figure 26A shows a pit in which the secondary wall and the warty layer have already been formed. At this stage there is still cytoplasm left in the cell, and the margo appears solid throughout. Later, after the membrane is perforated the margo in cross section is seen to consist of only two thin layers of microfibrils with a dense annulus at the edge (Fig. 26B). This phenomenon was visible only with aldehyde-fixed sections. With permanganate, the membrane of a bordered pit swells and separates (see Fig. 14), and there is no difference in appearance of the sectioned margo before and after preparation.

A more complete discussion of the development of the bordered pit membrane is published elsewhere (107).

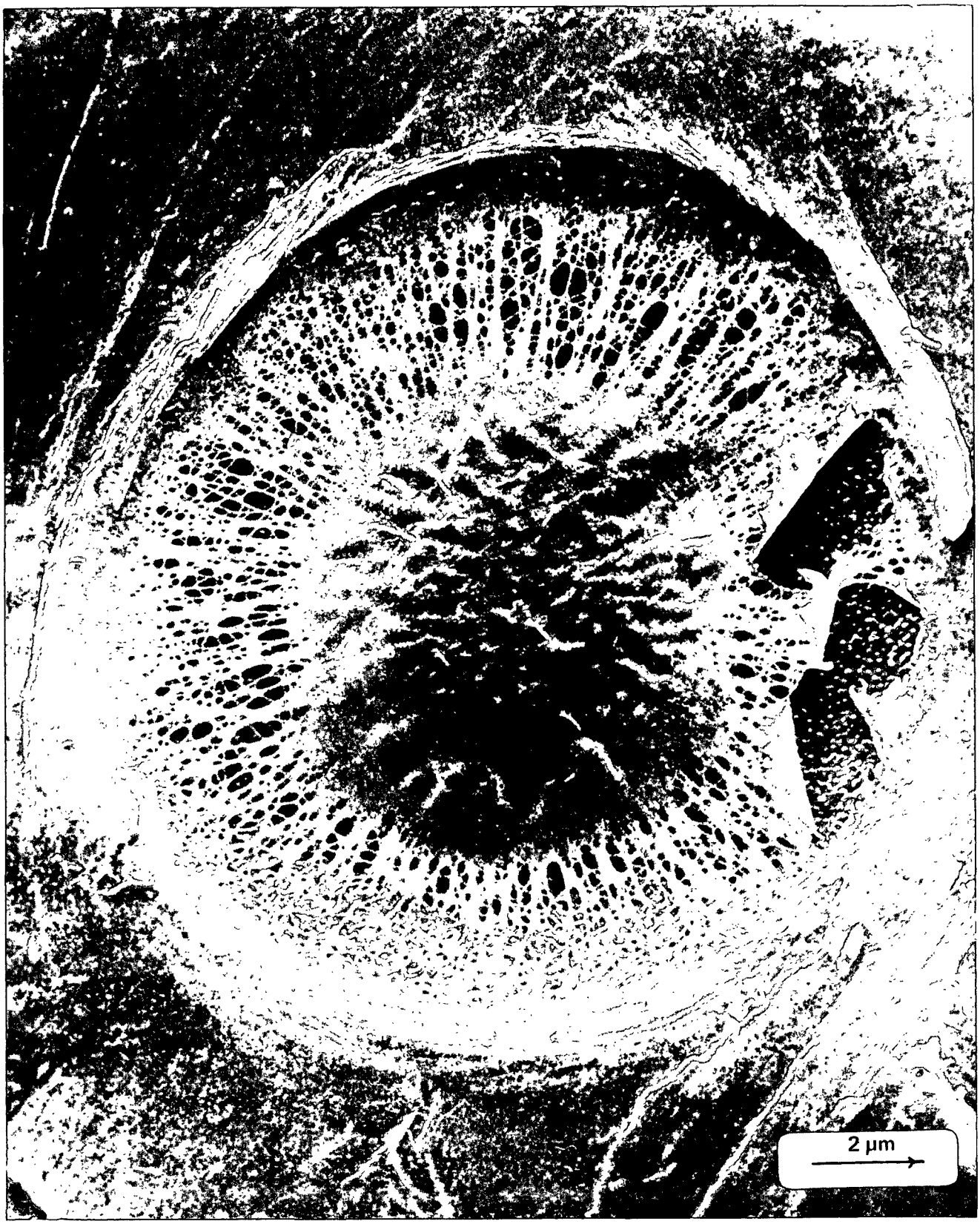


Figure 25. Mature, Perforated Membrane of Earlywood Intertracheid Pit; June 12 Sample, SEM Plate 829

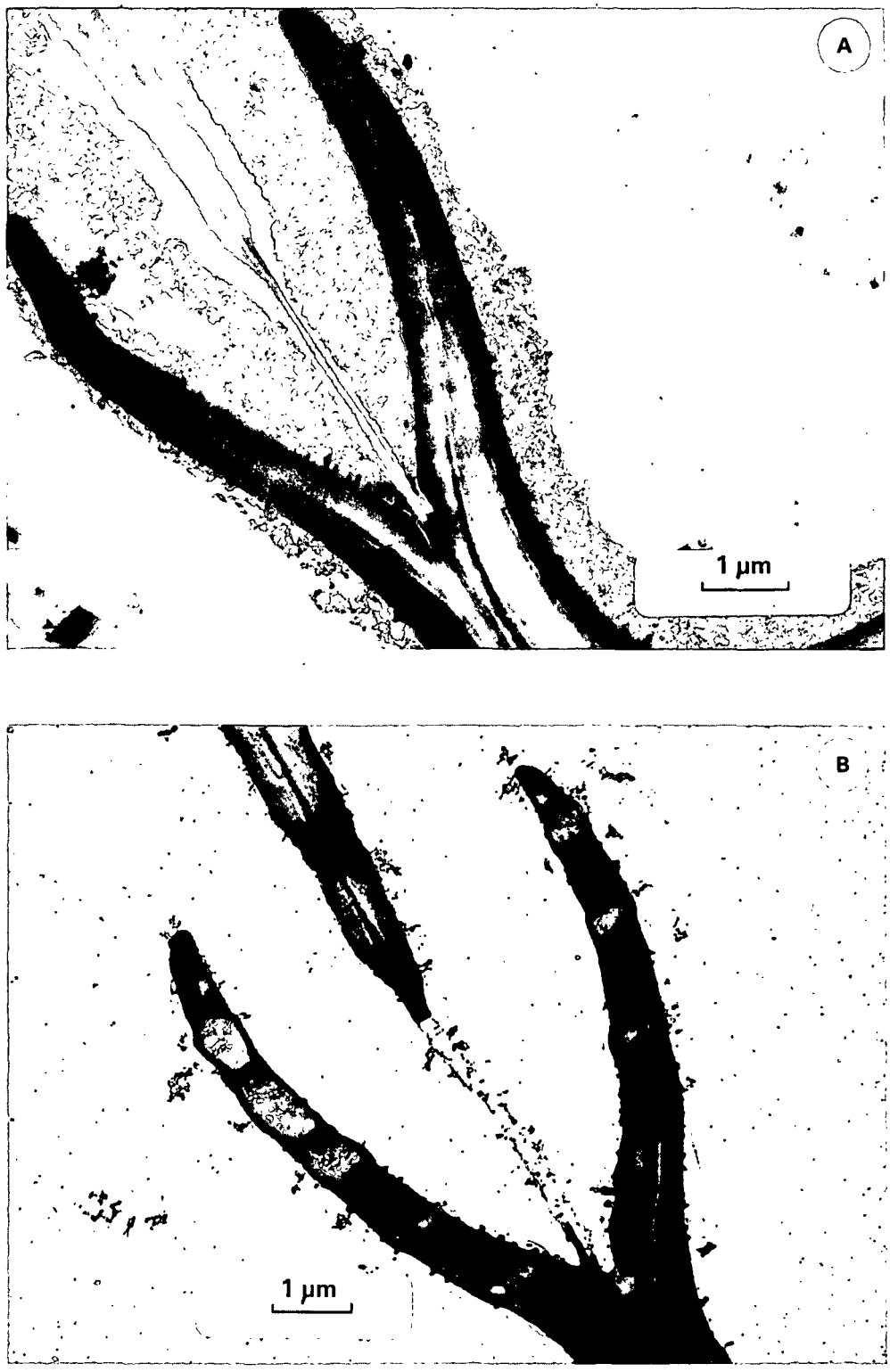


Figure 26A and 26B. Transverse Sections Illustrating the Perforation Process of the Intertracheid Pit Membrane:
A. Before Perforation; June 12 Sample, FAG-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 9740,
B. After Perforation; July 13 Sample, GAG-Fixed, Pb Citrate- and $KMnO_4$-Stained UTS, TEM Plate 10058

RESULTS OF TREATMENTS ON THE WARTY LAYER OF BALSAM FIR WOOD

The object in treating fir wood by different procedures and then assessing the effect on the warty layer was twofold. First, the response of the warty layer to various treatments gave some indication of the composition of this structure. The more specific the action of the treatment, the more precise the information that could be obtained. Second, if the warty layer was removed from the wood by a certain treatment, both the residual wood and the dissolved constituents were analyzed in an attempt to determine the chemical constitution of the warty layer. A problem here, however, was that since the warty layer makes up only about 2% of the total wood, the wart material often represented a minority of the total isolated material of a given treatment. Also, any treatment that dissolved the warty layer may have also altered it chemically, making determination of its native structure extremely difficult.

CHEMICAL TREATMENTS

The results of numerous chemical treatments on the warty layer of balsam fir are summarized in Table V. The effects caused by some of the treatments are shown in Fig. 27-42. With some exceptions, the results of these treatments were consistent with those of other workers presented in Tables IIIA and IIIB. Treatments found by previous workers to remove the warty layer but that had no effect on balsam fir warts were phosphoric acid (12) and neutral solvent extraction (42). Generally, however, balsam fir warts were found to exhibit the same reactivity as the warts of other species, both softwoods and hardwoods.

TABLE V

ACTION OF CHEMICALS ON THE WARTY LAYER OF BALSAM FIR

Treatment	Action
Soxhlet extraction with neutral solvents	No detected effect
Dioxane, room temp., 6 days	No detected effect
Dioxane, 60°C, 24 hr	No detected effect
Dimethylformamide, 125°C, 27 hr	No detected effect
Phenol, 90°C, 21 hr	No detected effect
Phosphoric acid, room temp., 3 hr	No detected effect
Chromic acid-nitric acid, room temp., 1½ hr	Amorphous supporting layer is removed exposing the S3, warts present but smaller, appear as low mounds (Fig. 29)
Sulfuric acid, room temp., 35 hr (surface of wood embedded with polystyrene)	Amorphous layer present, warts slightly smaller (Fig. 33)
DMSO-HCl, all 150°C	
0.1% HCl, 3 hr	Warts present but perhaps smaller
0.1% HCl, 5 hr	Warts removed in some areas, present but smaller in others
0.5% HCl, 3 hr	Warts completely removed from the lumen surface, small grains remain (Fig. 30)
H_2O_2-Acetic acid, all 2 hr	
5% 16.7%, room temp.	No detected effect
15% 0%, room temp.	No detected effect
7.5% 25%, 45°C	No detected effect
15% 50%, 45°C	Warts unchanged, some of the amorphous layer may be dissolved
7.5% 25%, 90°C	Warts and amorphous layer partially dissolved (Fig. 31A)
15% 50%, 90°C	Warts and amorphous layer completely dissolved (Fig. 31B)
30% 0%, 90°C	Warts and amorphous layer completely dissolved
Chlorite delignification, pH 4.0, room temp., 4 weeks	Amorphous layer extensively dissolved, residual wart material present as flat mounds (Fig. 32)

TABLE V (Continued)

ACTION OF CHEMICALS ON THE WARTY LAYER OF BALSAM FIR

Treatment	Action
Sodium hydroxide-sodium sulfide, 60°C, 2½ hr	No detected effect
17.5% Sodium hydroxide, 60°C, 2½ hr	No detected effect
0.1N Potassium hydroxide, 90°C, 2 hr	Warts and amorphous layer slightly dissolved, warts lying down (Fig. 28)
Digitonin, room temp., 11 days	No detected effect
Urea, room temp., 6 days	No detected effect
4-Methylmorpholine 4-oxide, 90°C, 22 hr	Cell wall indiscriminately eroded (Fig. 27)
Cadoxene, room temp., 4 days	Warts unchanged, pit margo dissolved
Quaternary ammonium base, 50°C, 22 hr	No detected effect
DMSO[a], 150°C	Warts extensively dissolved after 10 hr (Fig. 34A-34D)
Dioxane-0.5% HCl[a], 70°C	Warts extensively dissolved after 16½ hr (Fig. 36A-36D)
30% H_2O_2[a], room temp.	Warts extensively dissolved after 14 days (Fig. 38A-38D)
3% Peracetic acid[a], 60°C	Loblolly pine warts dissolved after 1 1/3 hr (Fig. 40A-40B); small mounds remain of balsam fir warts after 25 hr (Fig. 41)

[a]The results of these treatments are described more extensively in the text.

Several deductions can be made concerning the composition of the warty layer based upon the response of this structure to different chemical treatments.

Neutral solvents do not remove the warty layer, so it is not likely that wart structure consists of resinous deposits or other extractives. The digitonin treatment had no effect whatsoever. As a membrane-dispersing agent, it presumably would disrupt components found in a cytoplasmic membrane system.

The cellulose solvent, cadoxene, had no apparent effect on the warty layer, though the radiating microfibrils of the margo portion of the bordered pit membrane were dissolved. The tissue solubilizer, a quaternary ammonium base, also had no effect on the wart structure. 4-Methylmorpholine 4-oxide apparently eroded the entire wood structure including the warty layer (Fig. 27), but this chemical is not a specific solvent since it reportedly dissolves cellulose, hemicellulose, and lignin (87).

Treatments with concentrated alkali had no effect on the warty layer. The temperature for the treatments, 60°C, was probably not high enough for them to be effective delignifying agents (108). At higher temperature (90°C) and low alkali concentrations (0.1N), the warts were slightly affected (Fig. 28). Probably the major attack under these conditions occurs on the less resistant carbohydrates (109). Other investigators (46) have shown that the warty layer is removed under harsher conditions of kraft or soda pulping.

The following reagents can be classified as nonspecific, delignifying treatments: DMSO-HCl (110), chromic-nitric acid (111), acetic acid-hydrogen peroxide (111), and hydrogen peroxide alone (111). Under mild conditions these reagents can dissolve a portion of the warty layer, leaving small, flat

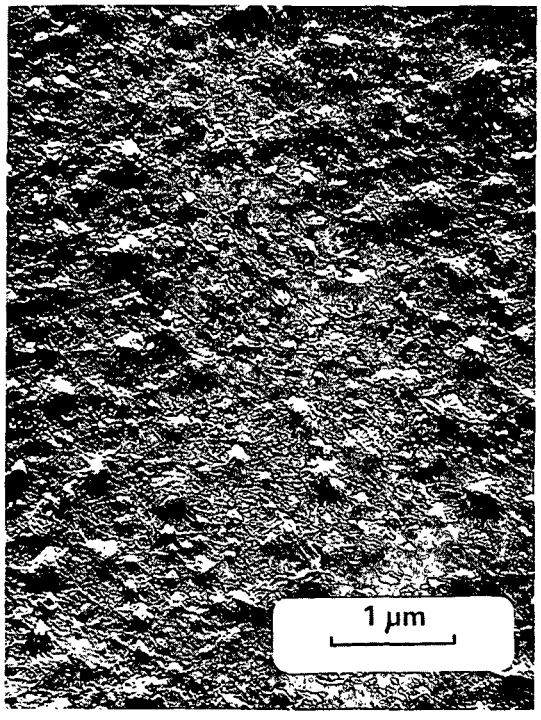


Figure 27. Mature Warty Layer Treated with 4-Methylmorpholine 4-Oxide at 90°C for 22 Hr; Replica, TEM Plate 10259

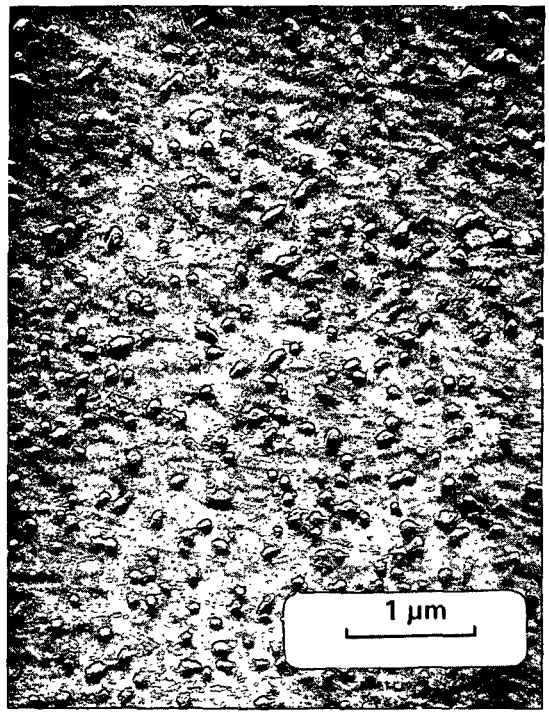


Figure 28. Mature Warty Layer Treated with 0.1N Potassium Hydroxide at 90°C for 2 Hr; Replica, TEM Plate 10119

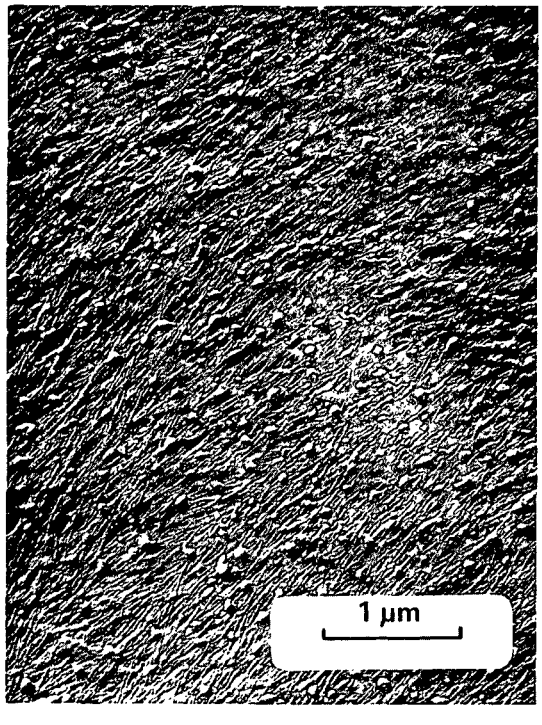


Figure 29. Mature Warty Layer Treated with 10% Chromic Acid-10% Nitric Acid (1:1) at Room Temperature for 3 Hr; Replica, TEM Plate 10256

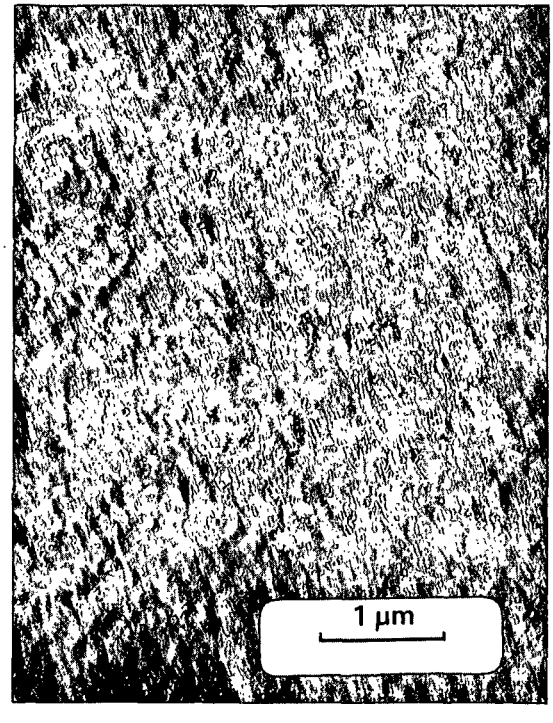


Figure 30. Mature Warty Layer Treated with 0.5% HCl in DMSO at 150°C for 3 Hr; Replica, TEM Plate 9443

mounds (Fig. 29, 31A). If harsher conditions are used, no wart remnants or S3 encrustants remain (Fig. 30, 31B).

The chlorite treatment is known to extract nearly all the lignin portion of wood while leaving the total carbohydrate fraction (86). Very small patches are left on the lumen surface and in many pit borders of fir chlorite holocellulose. The remnants are nearly flat with about the same diameter as the base of untreated warts (Fig. 32). The S3 layer here remains slightly encrusted, but the microfibrils are quite conspicuous. Interestingly, there is a similarity between the residual warts of fir holocellulose (Fig. 32) and the developing warts in differentiating tissue (see Fig. 21F).

Sulfuric acid shrinks the warts, but the amorphous layer and the protruding structures remain (Fig. 33). Treatment with this reagent is known to hydrolyze polysaccharides, leaving "Klason lignin" (112, 113). Some carbohydrate component from the interior of the wart structure may have been removed or the lignin portion may have been condensed by the treatment to cause the shrinkage. The majority of the wart structure and amorphous layer, however, are clearly ligninlike in composition.

Only by embedding the surface of wood sections in polystyrene before sulfuric acid treatment was the organization of the residue maintained and the ultrastructure of the lumen surface distinguishable. It is not surprising, therefore, that Scurfield and Silva (12) could not identify warts in wood hydrolyzed with sulfuric acid because of the considerable breaking of the unsupported cell wall. The polystyrene backing procedure used in this study does not shield the warty layer from the acid since the wart material was easily dissolved from the embedded section with sulfuric acid followed by chromic-nitric acid in the replica preparation procedure. It is difficult

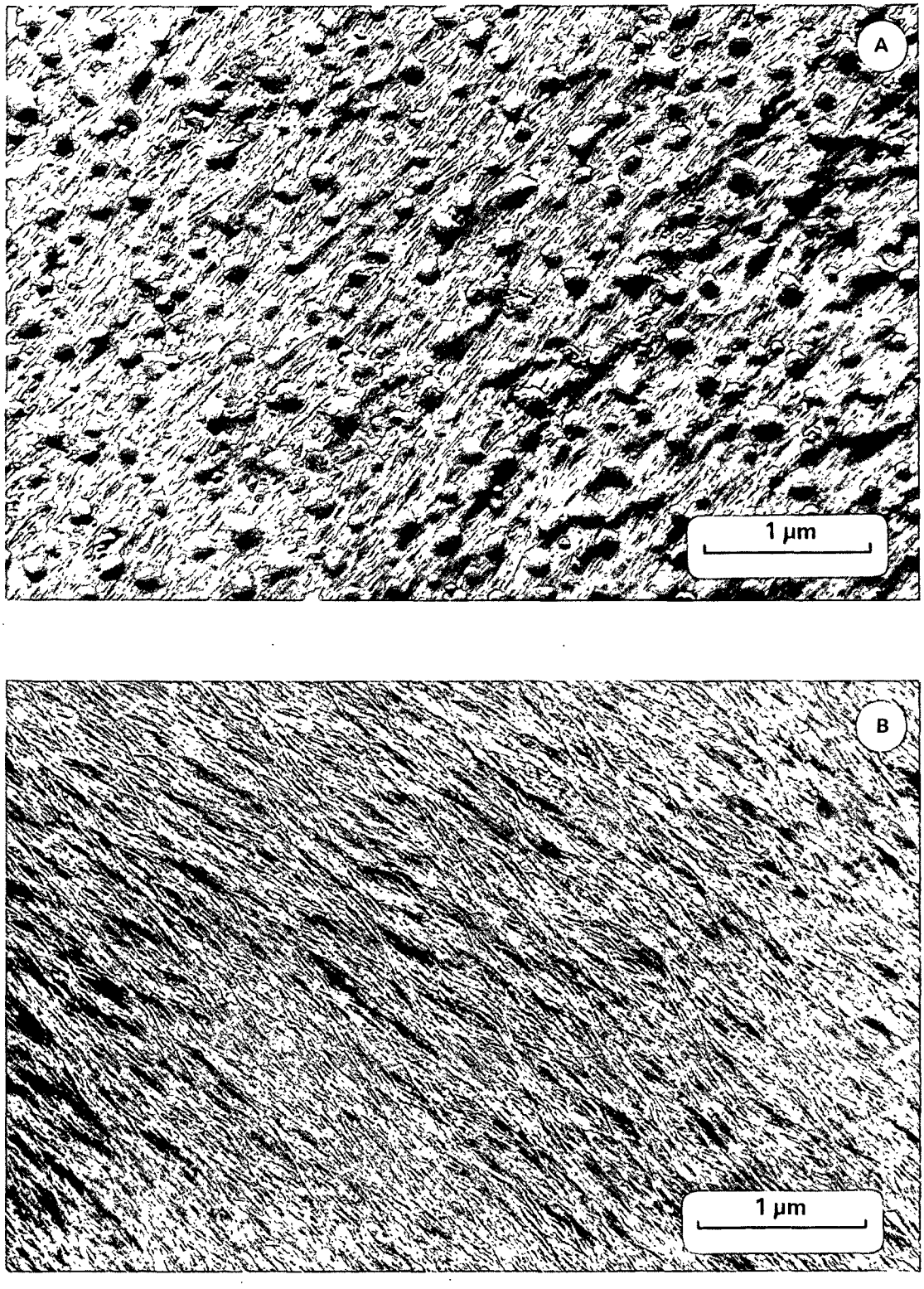


Figure 31A and 31B. Warty Layer Treated with Hydrogen Peroxide-Acetic Acid at 90°C for 2 Hr: A. 7.5% H_2O_2-25% Acetic Acid, B. 15% H_2O_2-50% Acetic Acid; Replicas, TEM Plates 9217 and 9216

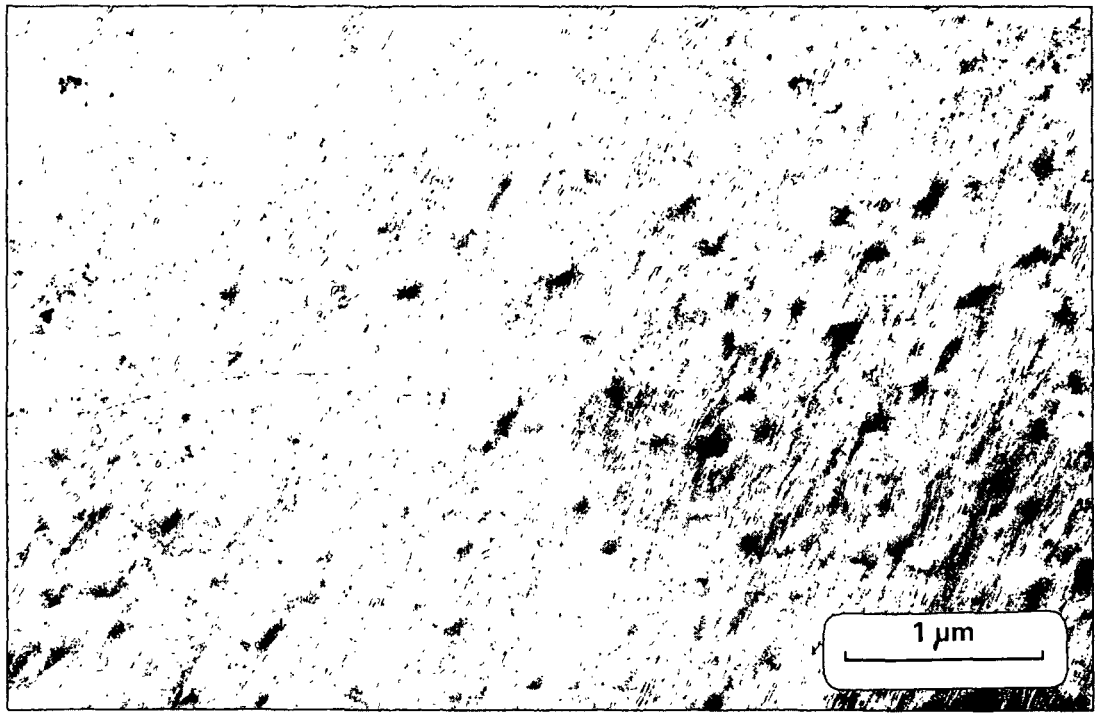


Figure 32. Warty Layer Treated with 4.5% Sodium Chlorite, pH 4.0, at Room Temperature for 4 Weeks; Replica, TEM Plate 10074

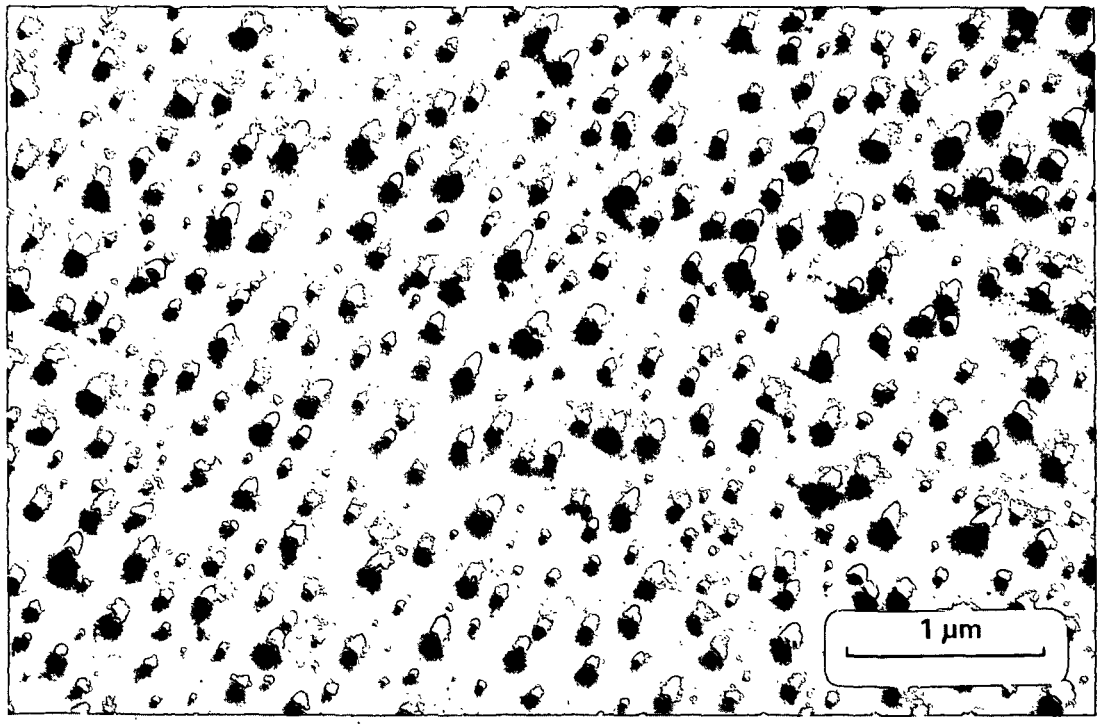


Figure 33. Warty Layer Treated with 72% Sulfuric Acid at Room Temperature for 35 Hr; Replica, TEM Plate 10083

to agree with Wardrop, et al. (16) that warts are unchanged after treatment with sulfuric acid. Some shrinkage definitely occurs (Fig. 33).

From the response of the warty layer to the different chemical treatments, it can be concluded that the major component of the warty layer is ligninlike. Some nonmicrofibrillar polysaccharide material likely makes up the basal component of the warts and the cell wall side of the accompanying amorphous layer. These latter features remain after chlorite delignification.

Four chemical treatments were studied more thoroughly to obtain information on the nature of the reactions that dissolve the warts. For each treatment, preliminary work was carried out to establish conditions necessary to extract the warty layer.

Dimethylsulfoxide (DMSO) Treatment

Dimethylsulfoxide is not a specific solvent for any single component in the cell wall. There are reports that it has been used as a solvent for hemicellulose (114) and lignin-carbohydrate complexes (115) and as an oxidant and solvent for lignin (110).

Action of Dimethylsulfoxide (DMSO)

When firwood is treated with DMSO at 150°C, the warts are removed after about 10 hr. Figures 34A-34D show the condition of the warty layer on the lumen surface at different stages of the DMSO extraction. After 5 hr of treatment, the amorphous layer and warts are still intact (Fig. 34A). The only change is that some warts are lying down. Such warts appear to coalesce with the amorphous layer and with each other. This action probably involves thermal softening and adhesive properties of the warts developed at the high temperature (116). After 10 hr of treatment the protruding warts are gone (Fig. 34B),

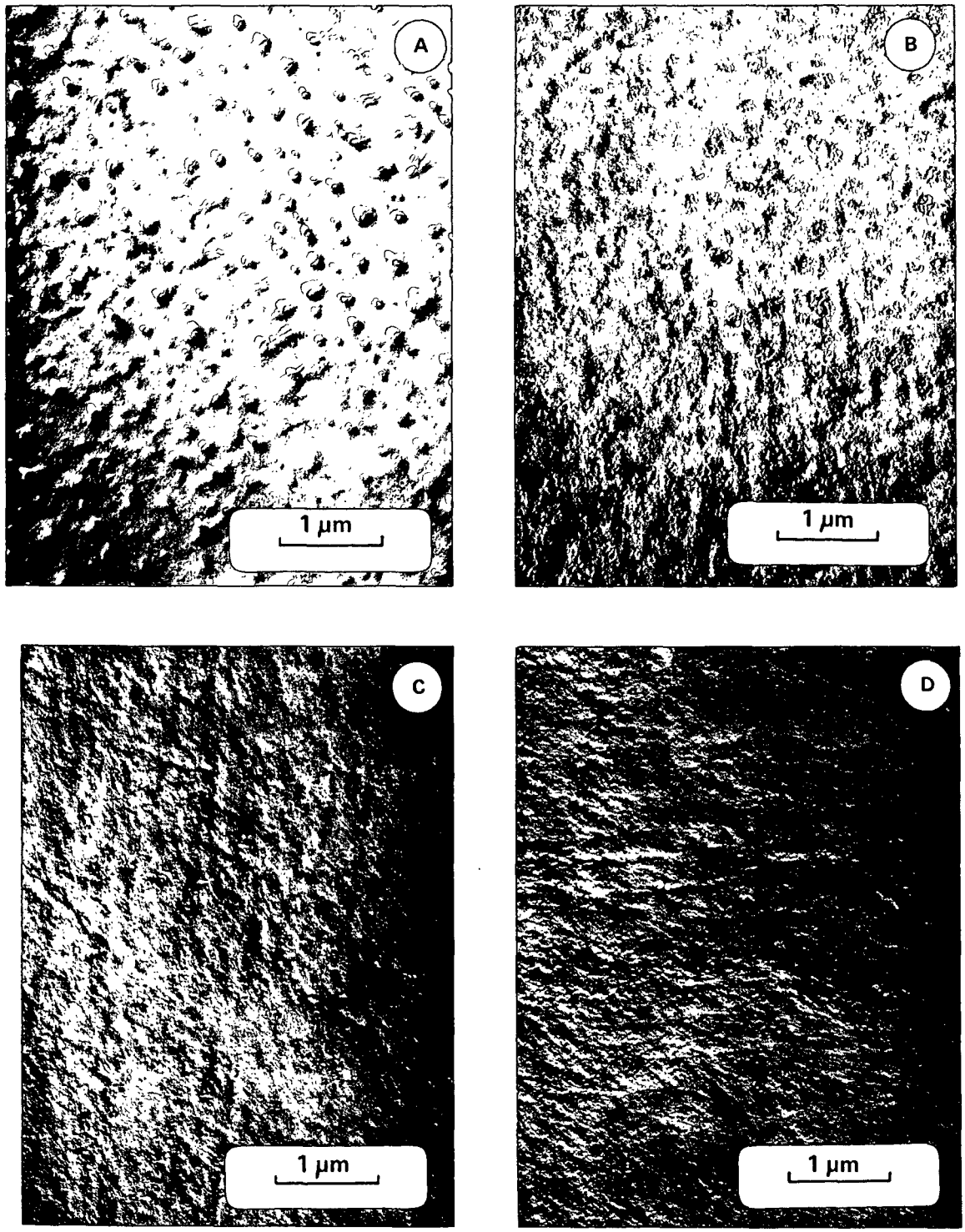


Figure 34A-34D. Stages in Warty Layer Treatment with DMSO at 150°C: A. 5 Hr, B. 10 Hr, C. 13½ Hr, D. 15 Hr; Replicas, TEM Plates 9506, 9500, 9462, and 9493

but a granular substance remains on the amorphous layer in locations where the warts were once present. This granular material was not extractable in boiling water. The amorphous layer is still intact covering the S3.

The warts are extensively removed in a surprisingly short time range in the period from about 9 to 11 hr. Generally, earlywood warts are removed slightly before latewood warts. Warts on the lumen-side of intertracheid pit borders are slightly less resistant than those on the rest of the cell wall, while those in the pit cavity are slightly more resistant.

By 13½ hr of treatment (Fig. 34C), much of the granular substance has been removed. The amorphous layer is still plainly present. After 15 hr (Fig. 34D), the remaining granular substance has been dissolved, and the microfibrils of the S3 are now faintly visible.

Prior extraction of the wood with neutral solvents had no effect on the rate or action of the DMSO solvolysis or on the results of other chemical treatments studied. Because of this circumstance, and since balsam fir is so low in extractive content anyway (117), wood samples were not usually extracted before treatment.

When the wood was oven-dried *in vacuo* at 105°C before DMSO treatment as it was here, the warts were removed in a slightly shorter time than if the wood was not dried (10 hr as opposed to 13 hr). Apparently, heating the wood under these conditions alters the structure or composition of the warty layer, making it more susceptible to DMSO dissolution. It is likely that oven drying *in vacuo* removes water of composition from wood constituents, thus forming condensation products. It is difficult to explain, however, why this would make the warts less resistant to the treatment.

The yield of firwood during DMSO treatment is given in Fig. 35. Klason lignin and apparent carbohydrate content determined by difference are also plotted. One drawback to Klason lignin determinations of treated wood is that some of the residual lignin in the wood may be modified by the treatment so that it is no longer insoluble in sulfuric acid. This would result in lower-than-actual values for the lignin content of treated wood. The Klason lignin content of balsam fir was 32.8% of the extractive-free, dry wood.

Consistent with the reported multiple-specificity of DMSO, the lignin and nonlignin portions of firwood are both extracted during the first 10 hr of treatment. Consequently, there are few clues to the composition of the warty layer from the analysis of residual wood. Still, an important observation that can be made is that while the warty layer is the most accessible portion of the cell wall to treatment solutions, it is not dissolved until 46% of the total lignin and 25% of the nonlignin part of the wood is dissolved (Fig. 35). Therefore, while the warty layer may be similar to lignin in its response to different treatments, it was more resistant to DMSO than about half of the lignin in wood. This difference in reactivity is probably due to a higher degree of condensation of the lignin molecule in the wart structure compared with lignin in the rest of the cell wall.

Analysis of Dimethylsulfoxide (DMSO) Extracted Material

Since so much material from the wood was solubilized by the DMSO treatment by the time the warts were removed, it was impossible to determine the composition of the warts by analyzing the total extraction solution. What was sought instead was any abrupt difference in properties between the extraction solutions immediately before and immediately after warts were removed.

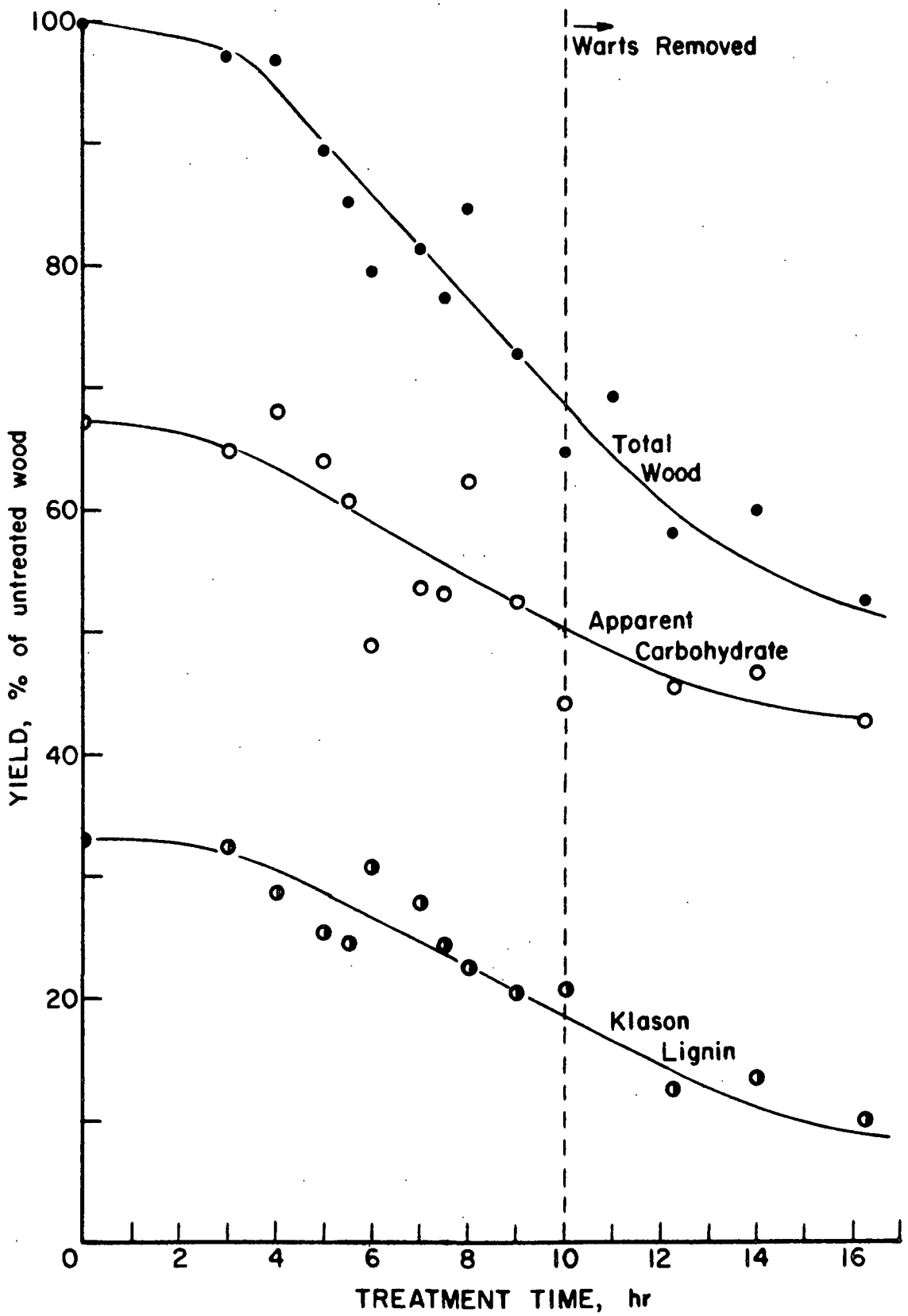


Figure 35. Yield of Balsam Fir Wood Treated with DMSO at 150°C

The same situation also existed for the dioxane-HCl extractions; consequently, subsequent analyses of the latter series followed the same strategy.

The pH of DMSO, both as a pure solvent and in a dilute aqueous solution, was slightly alkaline. During the first 6 hr of treatment, acidic groups were extracted from the wood, and the pH of the extraction solutions fell to a plateau value in the 3.5 to 4.0 range. Longer treatments resulted in no further drop in pH.

The UV absorbance spectra of the DMSO extraction solutions showed a definite peak at 277-278 nm and a shoulder at 315 nm. This absorbance is characteristic of lignin. The spectrum is likely a composite of absorption bands of different units that constitute the lignin polymer (118). The absorbance in the peak region is related to oxygen substitution on the aromatic ring of lignin. The absorbance at longer wavelengths appearing as a shoulder on the main peak can be attributed to the presence of carbonyl groups or double bonds conjugated with the benzene ring (118). DMSO alone is transparent only at wavelengths greater than 258 nm. The spectrum at lower wavelengths was, therefore, confounded by the extracting solvent.

Absorbance of the treatment solutions increased with longer extraction times as more lignin material was dissolved. However, there was no change in the general features of the spectra when warts were removed. It is known that the wart material absorbs UV light (16). Therefore, from the present work it can be concluded that the DMSO-dissolved wart structure has a UV absorbance similar to the extracted lignin, or if different, it is too dilute to be distinguished.

The refractive indices of the DMSO extraction solutions determined at 18.8°C increased gradually from 1.4790 for 0-hr treatment (pure DMSO) to 1.4794 for 14-hr treatment. There was no abrupt change in this property when dissolved warts entered the DMSO solution. A gradual increase in refractive index with longer treatments was not surprising since lignin has a measured refractive index of 1.61 (112) and that of warts has been estimated at 1.52 (16).

Centrifugation and ultracentrifugation of DMSO treatment solutions produced no sediment, indicating that warts were in fact dissolved and not merely freed during the treatment. Analytical ultracentrifugation of the 14-hr solution, which contained dissolved warts, gave a sedimentation coefficient of 0.18 s for the dissolved material in DMSO. This value is quite low compared with those for other isolated lignins (119), indicating that a substantial amount of low molecular weight material was present in the extraction solution. The 150°C, DMSO extraction was probably more than just a solvolysis of wood constituents since some depolymerization apparently occurred. Some extracted material could be precipitated from the DMSO solution with an excess of water, and the freeze-dried material was only partially soluble in dioxane, suggesting that high molecular weight material was also present.

Paper chromatography was used to isolate any low molecular weight lignin or carbohydrate components in the DMSO treatment solutions. The concentrated 7½- and 12¾-hr extractions were chromatographed in duplicate and detected. These solutions represented treatment durations before and after the warts were dissolved. The only clearly detectable substances found in the lignin developing system, Solvent IV, were at R_f values of 0.86, 0.61, and at the origin. Of these, the component at 0.61 appeared to be the most abundant.

The unknown component of greater mobility has an R_f value (0.86) and color reaction similar to known samples of vanillin (R_f = 0.85) and acetovanillone (R_f = 0.86) chromatographed simultaneously. The other component had an R_f value (0.61) in the range of ferulic acid (R_f = 0.57) and 4-hydroxy 3-methoxy-phenylacetic acid (R_f = 0.65) but a different color reaction from these known samples. The unknowns were detected in both extraction solutions using both detection reagents, 2,4-dinitrophenylhydrazine and diazotized p-nitroaniline; therefore, no unique material could be associated with the warts. From chromatogram color concentrations, there appeared to be slightly more fractionated material in the treatment of longer duration.

The same concentrated DMSO extraction solutions described above were also chromatographed with Solvent II to attempt identification of any sugars. No material migrated in either extraction solution, but there was material detected at the origin. Therefore, any carbohydrates present were probably in the form of polysaccharides.

Gas chromatography was used to analyze semivolatile components in the DMSO extractions. DMSO itself had a retention time of 3.4 min, which masked the area of the chromatogram between 2.8 and 6.0 min. The DMSO as supplied had three minor impurities with retention times on the column of 0.4, 0.5, and 9.0 min. The material at 9.0 min increased if DMSO alone was heated at 150°C. Two additional components were products extracted from the wood. Both increased in amount with longer extraction times and were not necessarily associated with the wart material. One of the latter products was volatile with a retention time of 1.8 min, indicating that it was definitely non-phenolic. It may be furfural, a carbohydrate degradation product with a retention time of 1.8 min also, under identical column conditions. The

other component had a longer retention, 11.5 min, which is in the range of phenolic compounds. Phenol itself had a retention time of 11.2 min; guaiacol, 7.0 min.

The technique of gel permeation chromatography was employed to approximate the molecular weight distribution of any lignin materials extracted with DMSO. The 7-, 8-, 12¼-, and 14-hr solutions were eluted with DMSO through a Sephadex G-25 column, which has an exclusion limit of 5000 for aqueous systems. Dissolved warts were present in the latter two treatments. The peak for a lignin sample of high molecular weight (91) identified the excluded volume and the beginning of the included volume. The peak for acetone identified the end of the included volume.

The elution properties of any solute in the fractionation range of a particular column can be described by a distribution coefficient, K_d, defined as the volume fraction of the included solvent in the gel particles that is available to the solute (90). K_d can be calculated from $(Ve-Vp)/Vi$, where Ve is the elution volume of the solute, Vp is the elution volume of excluded polymeric material, and Vi is the included volume.

On fractionation, all four of the above DMSO treatment solutions produced material in both the excluded volume (high molecular weight) and the included volume (low molecular weight). Division of this material between the excluded and included regions and the distribution coefficient of the included peak is given in Table VI.

The distribution coefficients of the included material, both before and after warts are extracted, are within the range of those for monomeric lignin components eluted in the same system by other workers (90). The

molecular weight of the excluded material here remains unknown other than presumably it is equal to or greater than the exclusion limit of the column, 5000.

TABLE VI

DISTRIBUTION OF MATERIALS IN DMSO EXTRACTION SOLUTIONS ELUTED THROUGH SEPHADEX G-25

Extraction Time, hr	Excluded Material[a]	Included Material[a]	K_d
7	1.9	1.1	0.71
8	2.1	1.0	0.79
12¼ warts removed	7.9	3.5	0.74
14	6.6	5.1	0.78

[a]Relative amounts of material calculated from area under UV-monitored elution curve and corrected for amount of wood extracted.

Over the period of time in which warts are dissolved by DMSO extraction (between the 8 and 12¼ hr), both low molecular weight and high molecular weight material increase appreciably. It is therefore impossible to discern from this work in what form (molecular weight) the wart material dissolved.

Dioxane-HCl Treatment

The warts of fir also can be removed by treating the wood at elevated temperatures with dioxane containing a small amount of concentrated hydrochloric acid. Such acidolysis is known to extract lignin from wood (112), causing in addition both depolymerization and condensation reactions (113). The action of the treatment, however, is not specific for lignin, judging from the fact that carbohydrate content may be substantial in extractions so obtained (121).

Action of the Dioxane-HCl

In a series of treatments of dioxane-0.5% HCl on firwood at 70°C, warts were found to be removed after about 16½ hr. Figures 36A-36D show the condition of the warty layer at different stages of this extraction.

Figure 36A shows the warty layer after 4½ hr of treatment. The warts are intact, but the amorphous layer is slightly dissolved, revealing some microfibrils of the underlying S3 layer. Up to this time there was no apparent alteration of the lumen surface. After 14 hr (Fig. 36B), the warts were partially dissolved, and the S3 orientation was quite visible, though some encrustant remained. Figure 36C shows the warty layer after 16½ hr with most of the wart material gone except for small mounds. The S3 orientation is visible as before. After 19 hr essentially all of the wart material was removed (Fig. 36D). Only small granular patches are evident where individual warts once were. The S3 layer is quite evident here with some remaining encrustant.

With extended dioxane-HCl treatment the condition of the lumen surface does not undergo much further change. Even after 56 hr, the small granular patches of residual wart material and the remaining S3 encrustant still persist.

The yield of firwood during dioxane-HCl treatment is given in Fig. 37. By the time the wart material is dissolved, 46% of the total wood substance is gone, including 65% of the Klason lignin and 38% of the apparent carbohydrate, even though the warty layer is the most accessible portion of the cell wall. It is apparent that this extraction favors but is not strictly specific for lignin, since during the first 5 hr of treatment significant carbohydrate extraction takes place. From that point on, extraction of carbohydrate continues but at a much slower rate. The rate of lignin

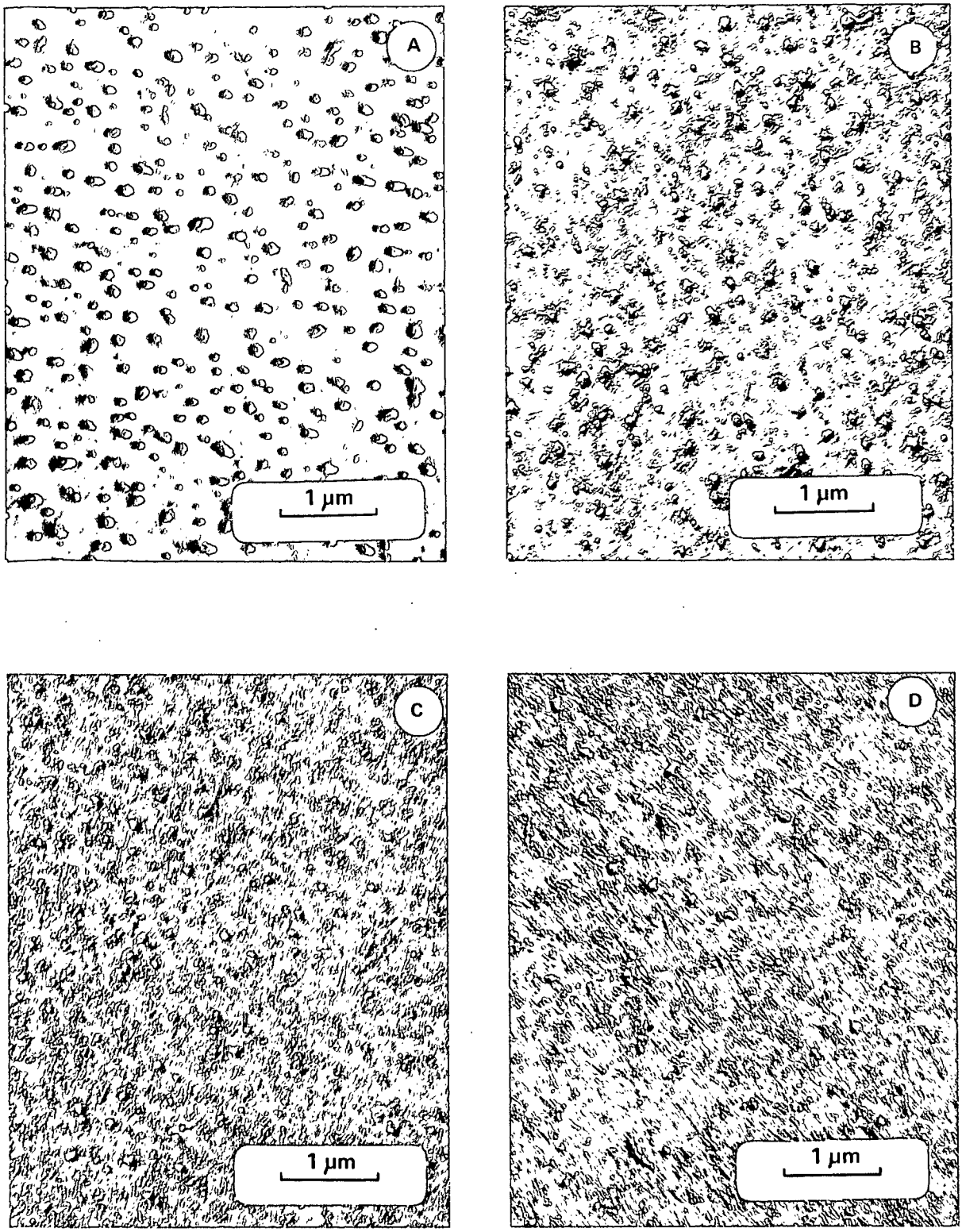


Figure 36A-36D. Stages in Warty Layer Treatment with Dioxane-0.5% HCl at 70°C: A. 4½ Hr, B. 14 Hr, C. 16½ Hr, D. 19 Hr; Replicas, TEM Plates 10248-10251

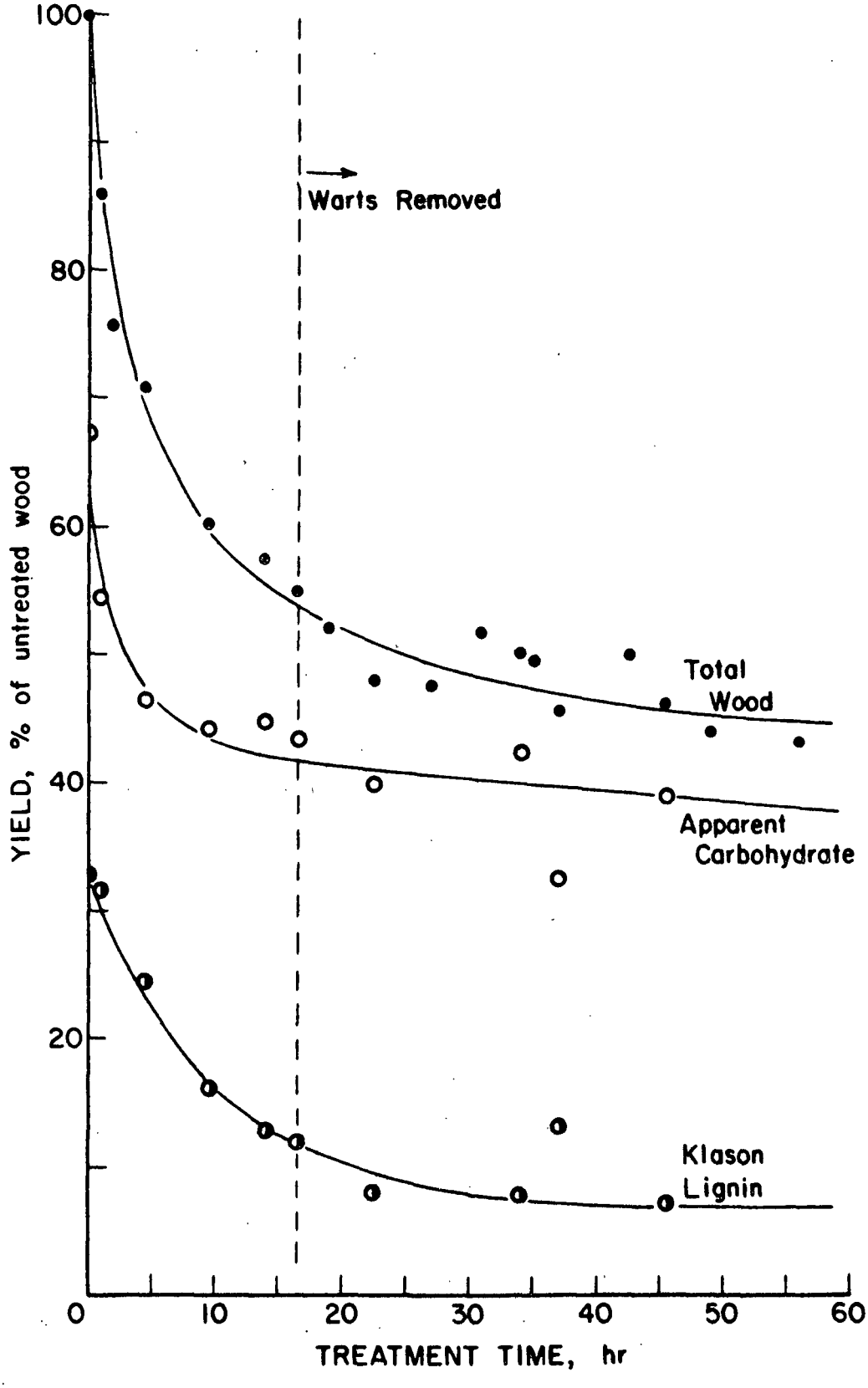


Figure 37. Yield of Balsam Fir Wood Treated with Dioxane-0.5% HCl at 70°C

extraction is greatest during the first 15 hr of the treatment; however, it also continues beyond this time at a slower rate.

The results presented in Fig. 37 are for wood vacuum-dried at 60°C before treatment. When the wood was vacuum-dried at 105°C, the warty layer was not dissolved, even after 60 hr of treatment. At 12 hr of treatment, the wood dried *in vacuo* at the higher temperature had a total wood yield of about 73%. Continued treatment resulted in no further weight loss. Evidently, vacuum drying at 105°C changes the composition of the warty layer and other wood constituents, rendering them resistant to the action of dioxane-HCl. A reasonable explanation is that water of composition is removed at 105°C *in vacuo* and not at 60°C. The condensation products thus formed at the higher temperature are then apparently more resistant to the dioxane acidolysis treatment.

Analyses of Dioxane-HCl Extracted Material

The pH of the dioxane-HCl extraction solutions was always below 0.5, indicating that acid was in excess even at the longest treatment times.

Within the transparent range of the solvent, the UV absorbance spectra of the dioxane-HCl extraction solutions showed peaks in the 269-277 nm range and shoulders at 315 nm. The maximum gradually shifted to longer wavelengths for the longer extraction times. This was perhaps due to an increase of material absorbing in the 315 nm range at longer treatment times. The ratio of absorbance at 315 nm to that of the main peak increased from 0.22 at 1 hr of treatment to 0.50 at 49 hr. Since the change in absorbance was gradual over the treatment sequence, it could not be attributed solely to wart material.

As with the DMSO treatment extractions, paper chromatography was also used here to isolate any low molecular weight lignin or carbohydrate material from the dioxane-HCl treatment. The concentrated 9½-, 14-, 19-, and 22½-hr extraction solutions were chromatographed in duplicate and detected. These solutions represent extractions before (9½- and 14-hr) and after (19- and 22½-hr) wart removal. The major spots in the Solvent IV development system for lignin monomers were at R_f values of 0.87, 0.72, 0.63, 0.54, 0.33, 0.24, 0.18, and at the origin. Of these, the component at 0.63 appeared to be most abundant. The component at R_f = 0.72 was detected only with 2,4-dinitrophenylhydrazine and the component at R_f = 0.54 only with diazotized p-nitroaniline. The other spots were detected with both reagents. The fastest moving unknown component had an R_f value (0.87) and color reaction similar to known samples of vanillin (R_f = 0.85) and acetovanillone (R_f = 0.86) chromatographed simultaneously. Components in the 0.55-0.65 R_f range had similar mobilities but different color reactions compared to 4-hydroxy 3-methoxyphenylacetic acid (R_f = 0.65) and ferulic acid (R_f = 0.57). Several other workers (122, 123) who have subjected dioxane acidolysis lignin to paper chromatographic analysis have detected several monomeric phenylpropane derivatives plus vanillin. All unknown components were found in all four extraction solutions and in about the same amount; therefore, no unique material could be associated with the warts.

The 14- and 22½-hr dioxane-HCl extraction solutions were chromatographed with Solvent II to identify any sugars present. Four fractionated components, plus material at the origin were detected in both solutions. Two of the components could be tentatively identified as glucose and mannose because of their identical mobilities and color reactions to known samples. The other two migrating components were likely disaccharides containing glucose or

mannose or both. Within each solution the amounts of the four fractionated components were nearly the same. There also was no obvious difference in amount of components obtained from the longer extraction time (with dissolved warts) compared to the short extraction time (without dissolved warts).

Gas chromatography was used to analyze semivolatile components in the whole set of dioxane-HCl extraction solutions. There was only slight quantitative variation in the chromatograms for the different extraction periods. There was no significant change in the pattern in those extraction solutions in which the warts were dissolved.

Several peaks were present in the aforementioned chromatograms in addition to solvent peaks at 0.5 and 0.6 min. Only two peaks, those at 1.4 and 19.2 min retention times, were products extracted from the wood. Both of these were very small peaks and both were extracted in the early hours of the reaction before the warts were removed, suggesting that low molecular weight components were extracted early and that the warts are removed as high molecular weight material. The origins of components representing the other peaks were determined by chromatographing several control solutions. The dioxane as supplied had a minor contaminant perhaps arising from the 0.001% sodium diethyldithiocarbamate added by the supplier as a stabilizer.

Components at 3.7, 5.2, and 12.8 min represented dioxane-HCl reaction products (or stabilizer-HCl reaction products) that were formed when dioxane and concentrated HCl stood at room temperature. They were present in neither the dioxane nor the HCl used to make up the solvent. These components may represent active species in the wood extraction because they were consumed during the reaction with wood but were not diminished when the solvent was heated alone.

Freudenberg (124) has suggested that the dissolution of lignin by dioxane-HCl is due to some contaminant present in the dioxane. He was unable to extract any lignin from sprucewood by heating it with a specially purified dioxane in the presence of 1-2% HCl at 110-120°C. While no confirmation of this suggestion was attempted in the present work, the findings here indicate that some reagent in addition to dioxane and HCl may be involved in the extraction.

The technique of gel permeation chromatography was used to approximate the molecular weight distribution of the dioxane-HCl extracted lignin material. The 14- and 22½-hr extractives were redissolved in DMSO and eluted through a Sephadex G-25 column. The longer treatment contained dissolved warts while the shorter treatment did not. Both treatment solutions had material in the excluded and included portions of the eluted volumes with definite peaks or shoulders in both regions. The division of material between the excluded and included regions and the distribution coefficient of the included peak as defined earlier are reported in Table VII.

TABLE VII

DISTRIBUTION OF MATERIALS IN DIOXANE-HCl EXTRACTION SOLUTIONS ELUTED THROUGH SEPHADEX G-25

Extraction Time, hr	Excluded Material[a]	Included Material[a]	K_d
14	7.7	6.9	0.72
22½ warts removed	12.0	6.5	0.70

[a]Relative amounts of material calculated from area under UV-monitored elution curve and corrected for amount of wood extracted.

The distribution coefficients for the included material, like those for the DMSO extracted material, are consistent with the values for monomeric lignin components eluted from the same system (90).

Over the period of time in which the warts are extracted, the low molecular weight material in solution does not change appreciably; therefore, it appears that warts are in fact extracted by dioxane-HCl as high molecular weight material.

Hydrogen Peroxide Treatment

The warty layer of fir can be removed with 30% H_2O_2 at room temperature. This reagent is a strong oxidant. At elevated temperatures the lignin macromolecule can be extensively degraded and dissolved by peroxide with the primary attack directed at the aromatic nuclei (111). At room temperature, however, even prolonged treatments with H_2O_2 solution causes very little degradation of lignin. Still, methylene groups may be converted into carbonyl groups and these, in turn, are susceptible to conversion to carboxyl functions (111).

Most warts were removed from the lumen surface by 14 days of treatment with 30% H_2O_2 at room temperature. With the DMSO and dioxane-HCl treatments, the warts were all dissolved over a relatively short period of time and variation of wart structure from tracheid to tracheid was not great for a given treatment time. With the H_2O_2 treatment, however, wart dissolution was never uniform over a given wood section for a given treatment time. After 7 days of treatment the warts and amorphous layer were completely dissolved in some places while in others the warty layer was apparently unchanged. By 14 days most of the warts were dissolved, but there was still quite a range of action. Small oxygen bubbles, which formed on the wood sections, may have protected some areas and caused the inhomogeneous response.

Figures 38A-38D show the typical condition of the warty layer at different stages of the H_2O_2 extraction. After 7 days of treatment (Fig. 38A), the warts are smaller but still distinct. The amorphous layer remains. In some areas it appears that the partially dissolved warts have a doughnut morphology (Fig. 38B). Here, it could be that the interior parts of the warts were oxidized first by the peroxide, leaving small craters. Figure 38C shows the action of the 11-day treatment. The warts are reduced to flat patches, but again the amorphous layer remains intact. By 14 days the warts are extensively removed (Fig. 38D). The S3 layer is still obscured by an encrustant, but with still longer treatments, the amorphous layer can also be dissolved consistently from every tracheid.

The yield of firwood during treatment with 30% H_2O_2 at room temperature is plotted in Fig. 39. By 14 days when the warty layer is extensively dissolved, 20% of the total wood is extracted, including 16% of the Klason lignin and 22% of the apparent carbohydrate material. The peroxide attacked both lignin and carbohydrate at similar rates, although neither was attacked very rapidly under the conditions used. With H_2O_2 the warts were removed at an earlier part of the extraction, in terms of amount of material dissolved, when compared with the DMSO and the dioxane-HCl treatments.

The results presented in Fig. 39 are for wood dried at 60°C in vacuo before treatment. When the wood was dried at 105°C in vacuo there was no apparent effect on the ultrastructure of wood, even on prolonged H_2O_2 treatment. The wood yield remained unchanged at about 92% beyond 6 days of treatment. Vacuum drying at 105°C rendered the warts and other wood constituents resistant to oxidation by peroxide just as it made them resistant to acidolysis by dioxane-HCl. This phenomenon did not occur at 60°C. Again, a possible explanation is that resistant condensation products may be formed at the higher drying temperature when water of composition is driven from the wood.

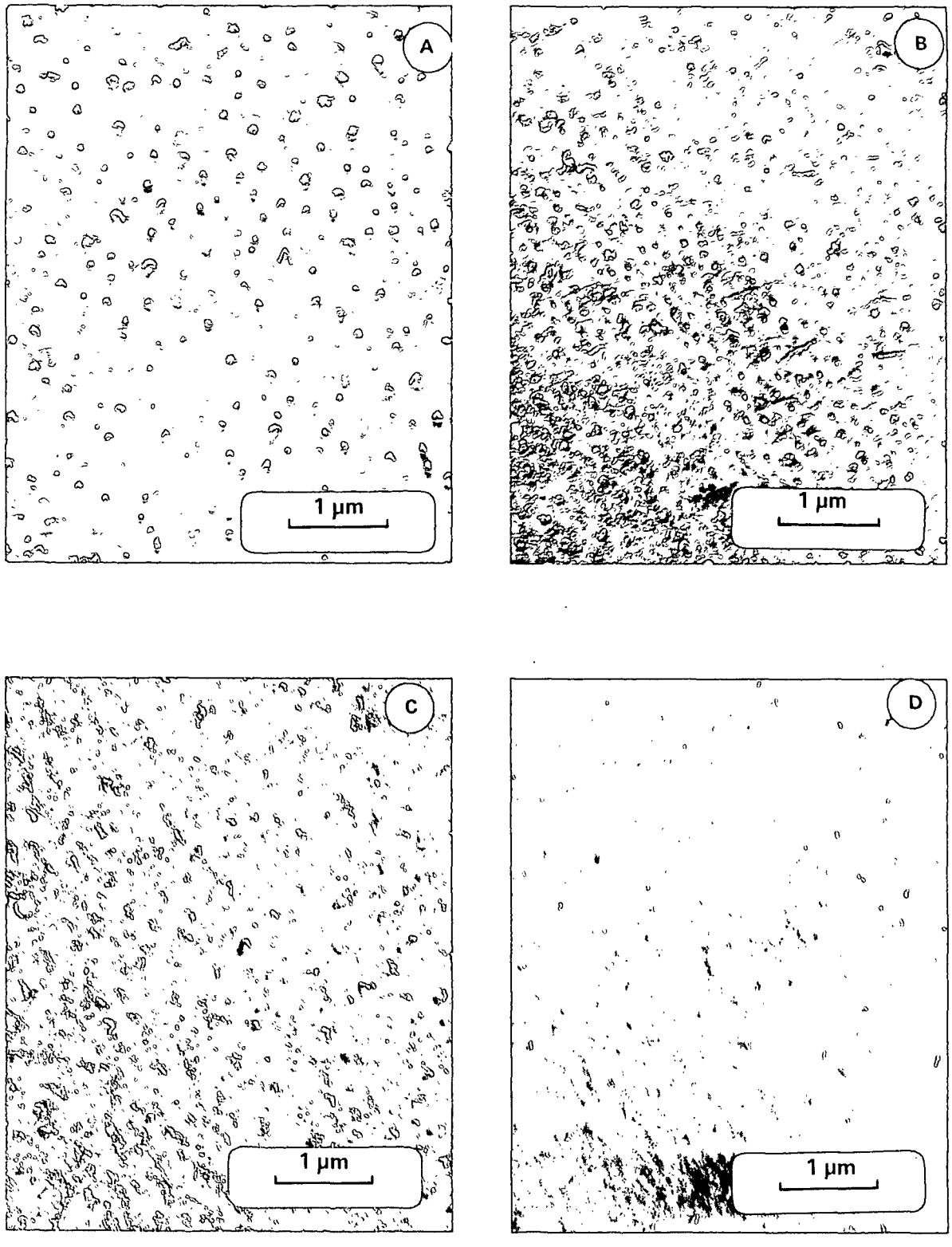


Figure 38A-38D. Stages in Warty Layer Treatment with 30% Hydrogen Peroxide at Room Temperature: A. 7 Days, B. 7 Days, "Doughnut" Morphology, C. 11 Days, D. 14 Days; Replicas, TEM Plates 10252-10255

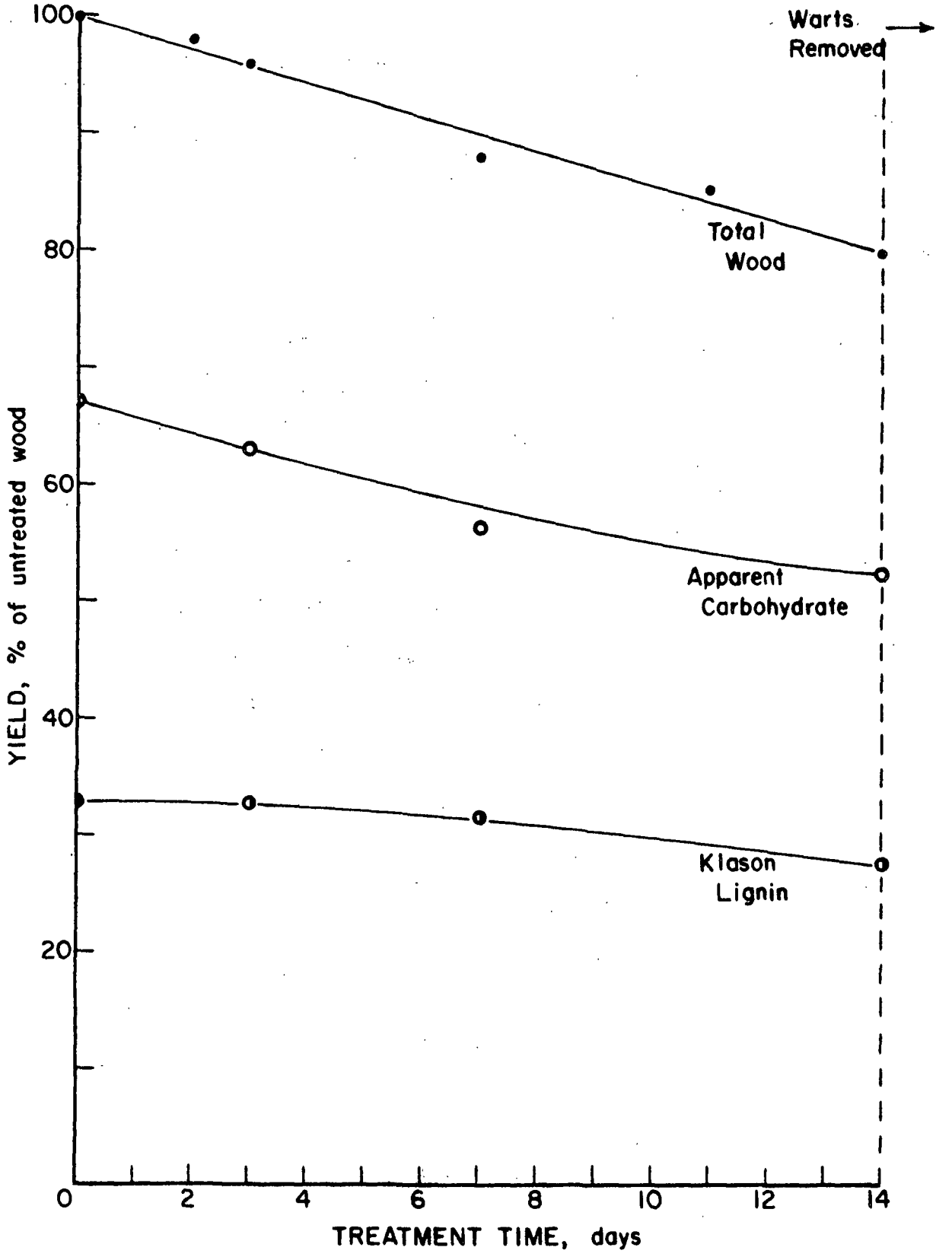


Figure 39. Yield of Balsam Fir Wood Treated with 30% Hydrogen Peroxide at Room Temperature

Since the entire warty layer was not dissolved uniformly over a short time range, further analyses of the peroxide treatment solutions were not attempted. In any event, it would have been difficult to determine the useful UV spectrum of the treatment extractives because 30% H_2O_2 itself absorbs very strongly below 330 nm.

Peracetic Acid Treatments

Two species of wood, balsam fir and loblolly pine, were treated with 3% peracetic acid at 60°C. In experimental softwood pulping studies this reagent has been shown to be highly specific in its attack upon lignin with only small losses of hemicellulose (125).

Figures 40A and 40B show the lumen surface of loblolly pine tracheids before and after treatment with peracetic acid, respectively. The untreated warts of this species appear as low mounds lying directly on the microfibrillar S3 layer with very little or no additional encrustant (Fig. 40A)*. After 80 min of treatment with peracetic acid, warts were removed from the lumen surface of loblolly pine (Fig. 40B). Warts in the pit borders of this species were somewhat more resistant, but they also were extensively dissolved by 4 hr of treatment. Albrecht (84) found that the initial solubilized reaction products of peracetic acid on loblolly pine were largely low molecular weight lignin fragments.

With balsam fir, the warts are not dissolved until after 25 hr of treatment with peracetic acid (Fig. 41). Even here remnants of the wart structure remain though the major part of the amorphous layer is removed. As with the

*Note the similarity between mature, untreated loblolly pine warts and the immature warts in differentiating fir (Fig. 21F).

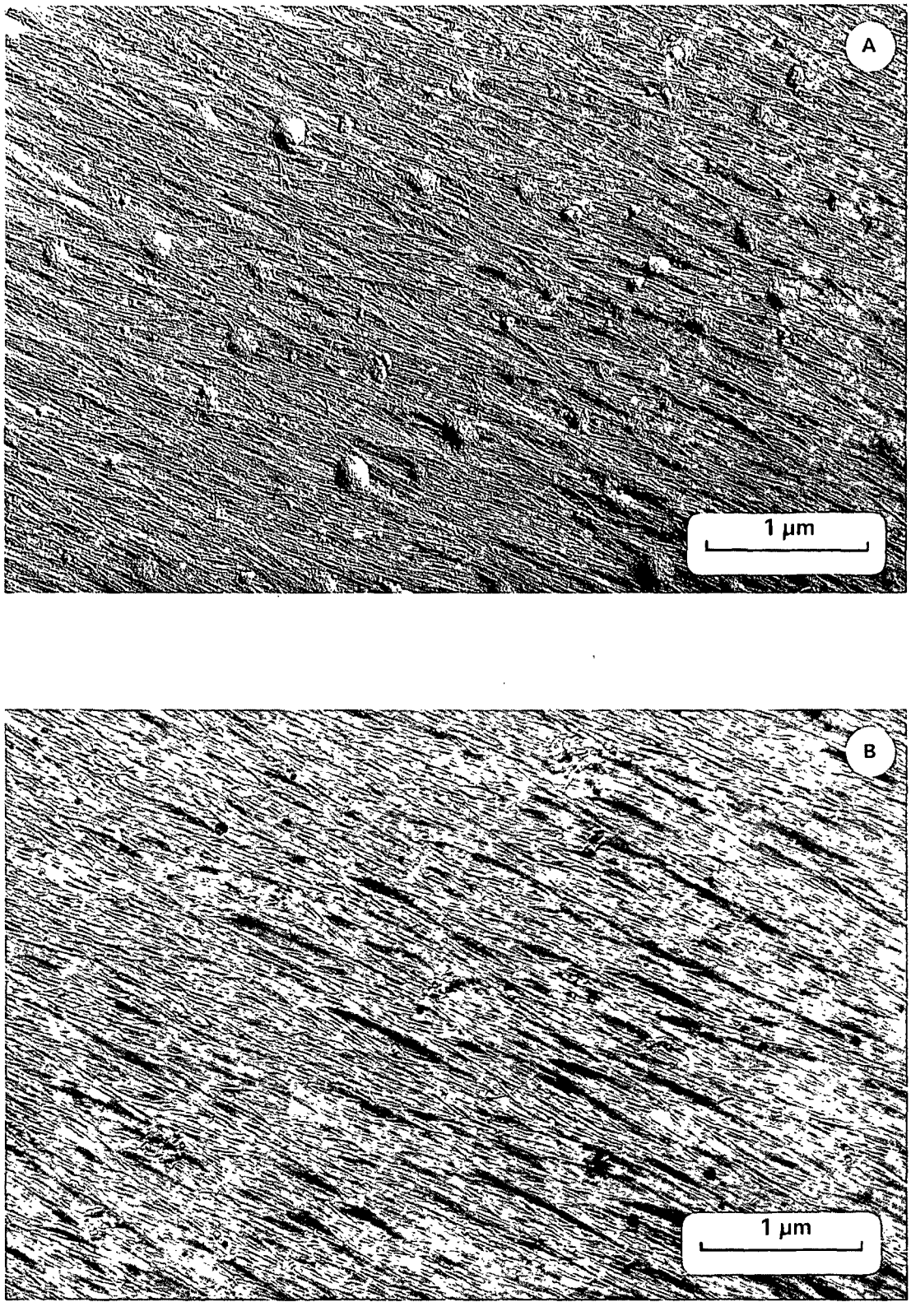


Figure 40A and 40B. Warty Layer of Loblolly Pine: A. Before and B. After Treatment with 3% Peracetic Acid at 60°C for 80 Min; Replicas, TEM Plates 9571 and 9548

chlorite delignification, the remnant material is probably carbohydrate. Therefore, in addition to being different in structure, the warts in balsam fir were different in their response to treatment with peracetic acid. The warts in loblolly pine were among the first wood components removed by the treatment, while the warts in fir were still partially present even after prolonged treatment. This difference in reactivity can be explained by suggesting that the lignin portion of the warty layer in balsam fir is more highly polymerized than the lignin in the warts of loblolly pine. Also, the warts in this latter species very likely have little or no carbohydrate component.

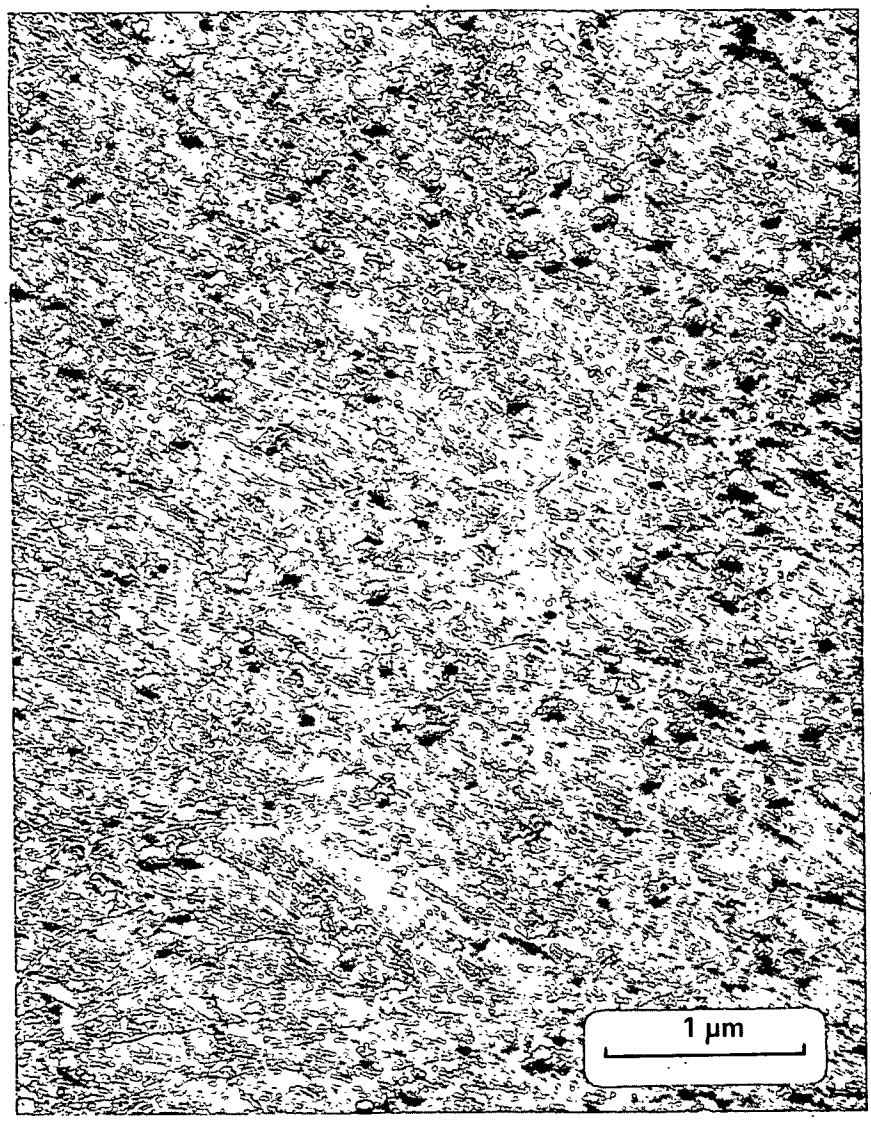


Figure 41. Warty Layer of Balsam Fir Treated with 3% Peracetic Acid at 60°C for 25 Hr; Replica, TEM Plate 10023

The yields of fir- and pinewood during treatment with peracetic acid are plotted in Fig. 42. By the time the warts are extensively removed from fir, 26% of the wood is dissolved, including 55% of the Klason lignin and 12% of the apparent carbohydrate. The specificity for lignin in this reaction is obviously high, as reported, especially during the first stages of the reaction.

With peracetic acid, delignification is much slower for fir than for pine. The nature of the innermost cell wall layer may be responsible for this difference. Wood structure is most easily accessible to treatment solutions through the empty cell lumen. The innermost lamella of pine is an open, microfibrillar structure with flat warts directly on the S3 layer (see Fig. 40A). In fir, on the other hand, the S3 layer is completely covered by a very resistant amorphous layer in addition to the warts (see Fig. 4). It is quite reasonable to propose that this amorphous layer is highly condensed and acts as a barrier to the penetration of peracetic acid into the cell wall, contributing to the reduced rate of delignification in fir.

ENZYMATIC TREATMENTS

Many pure enzymes would be ideal treatment agents because of their strict specificity; however, none of the commercial enzymes listed in the Materials and Methods had any effect on the structure of the warty layer of mature, untreated firwood. Peroxidase and tyrosinase in combination with an alkali pre- or posttreatment of the wood also had no apparent effect.

Pectinase had no apparent effect on the warts of untreated loblolly pine or balsam fir. The enzyme did, however, catalyze the dissolution of the remnants of the warty layer in fir remaining after chlorite delignification. The normal structure of the lumen surface in fir chlorite

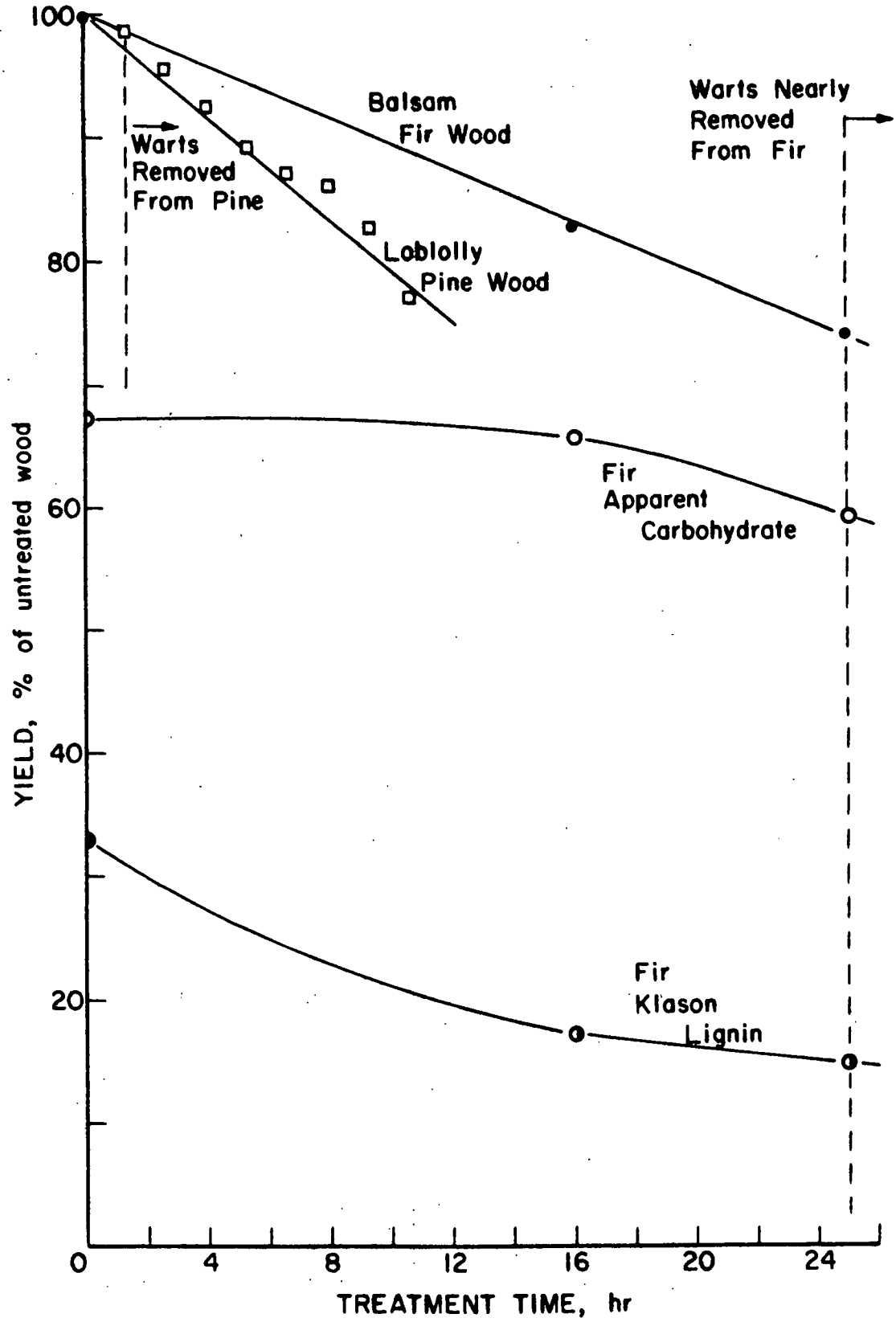


Figure 42. Yield of Balsam Fir and Loblolly Pine Wood Treated with 3% Peracetic Acid at 60°C

holocellulose is shown in Fig. 32. After pectinase treatment, the patchlike wart remnants and the remaining S3 encrustant are completely dissolved (Fig. 43). Another effect of the enzyme was to dissolve some intercellular substance that served to bind the delignified fir tracheids together. After pectinase treatment the tissue separated more easily into individual fibers. This is not surprising since the intercellular region has been shown to contain a high percentage of pectic material (126). An important consideration in the subsequent analysis of the pectinase-holocellulose hydrolyzate, therefore, is that material in addition to the dissolved wart remnants is present.

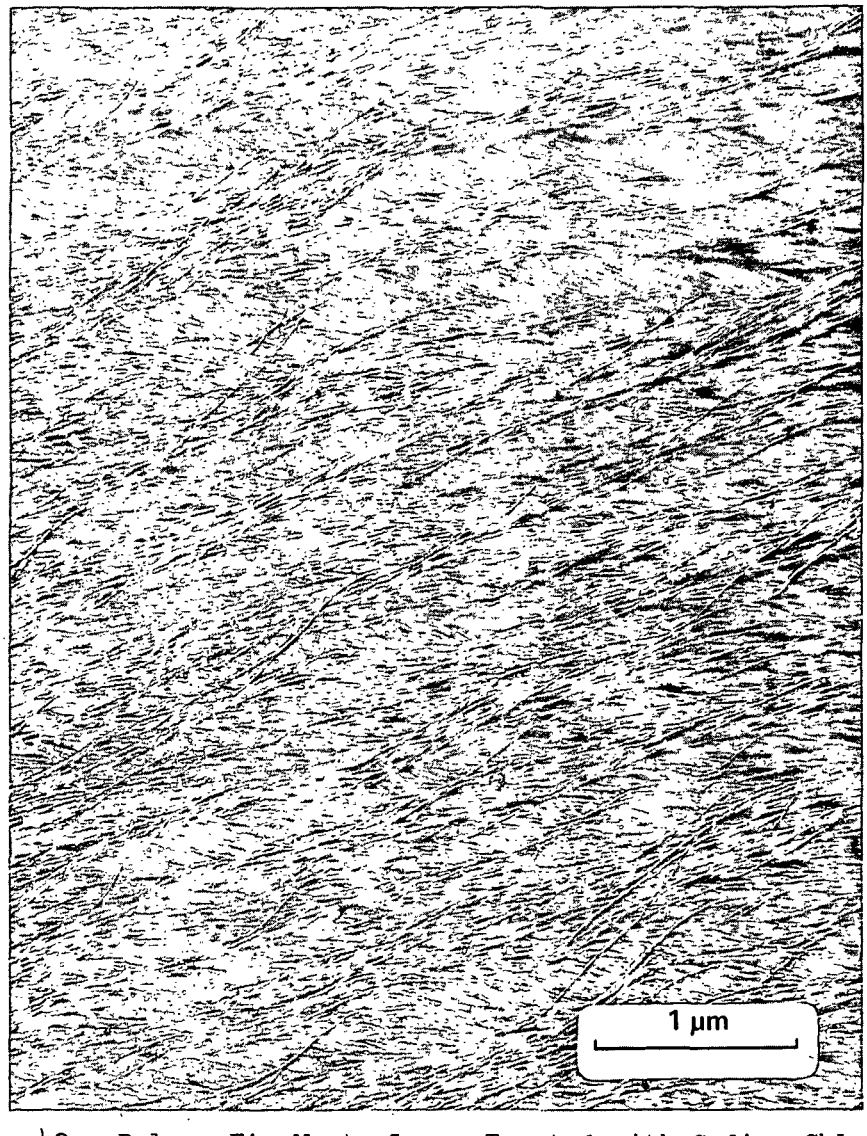


Figure 43. Balsam Fir Warty Layer Treated with Sodium Chlorite then Pectinase; Replica, TEM Plate 10124

Timell (89) reported that the same pectinase enzyme preparation as used in the present work had wide specificity, hydrolyzing the following polysaccharides with formation of mono- or oligosaccharide products: an arabino-4-O-methylglucuronoxylan, a hardwood glucomannan, a water-soluble and an alkali-soluble softwood galactoglucomannan, an ivory nut mannan, citrus and bark pectin, and starch. Cellulose suffered hardly any breakdown. Because of this wide range of enzyme specificity, all that can be concluded before further analysis of the hydrolyzate is that the wart remnant material left in fir chlorite holocellulose likely consists of a noncellulosic polysaccharide.

Thin-layer and paper chromatography were used to analyze the products in the pectinase-holocellulose hydrolyzate. Thin-layer chromatography with Solvent I showed the following components in the hydrolyzate: xylose, arabinose, mannose, glucose, and galactose, each estimated to be about 0.5-1.0% of the original holocellulose. These components were tentatively identified by comparison with the color reaction and mobility of known sugars run simultaneously. Amounts were estimated by comparison with the visual intensity of known concentrations. In addition to these named components, at least two slower moving components were also fractionated but could not be identified from knowns.

Paper chromatography with both Solvents II and III confirmed the presence of the same aforementioned neutral sugars. These were present in nearly equal amounts with mannose slightly more and glucose slightly less than the rest. From control chromatograms it was determined that some of the glucose was actually from the enzyme itself, being incompletely removed by the dialysis purification procedure. Therefore, the amount of glucose from the holocellulose may be considerably less than the other sugars. Other components were fractionated but not positively identified. There were fractionated

spots of low mobility that gave color reactions of pentosans or uronic acids. These materials were present in amounts similar to the neutral sugars. Reference compounds indicated that the following components were not present in the hydrolyzate: galacturonic acid, glucuronic acid, 4-O-methylglucuronic acid, aldobiuronic acid, or aldotriuronic acid. Components possibly present included di- and triuronic acids, aldotetrauronic acid (or higher oligomers), esterification products of uronic acids with themselves, or pentosans.

The pectinase-holocellulose hydrolyzate, which contained dissolved wart remnants, gave a negative test for characteristic lignin groups with 2,4-dinitrophenylhydrazine (120) and diazotized p-nitroaniline (127).

The pectinase dissolution of the wart remnants after chlorite delignification and the subsequent analyses indicate that this material is most likely pectin or a pentosan-containing hemicellulose. This is consistent with the results of Meier (126) who found that glucuronoarabinoxylan content is very high in the S3 layer of softwoods.

FUNGAL TREATMENTS

Six different species of white-rot fungi* were investigated with regard to their effects on wood ultrastructure. White-rot fungi degrade lignin, hemicellulose, and sometimes at a later stage, cellulose (128). It has been shown that different white rots vary considerably in the comparative rates of carbohydrate and lignin degradation (130). The species of fungi used in

*Liese (128) distinguished between white-rot fungi, which preferentially attack lignin and hemicellulose, and "simultaneous-rot" fungi, which decompose all substances of the lignified cell wall. In the present discussion, these two types are both considered as white-rot fungi as is traditionally done (129, 130).

the present work and their action on firwood sections, as observed with the TEM and SEM, are presented in Table VIII.

TABLE VIII

ACTION OF WHITE-ROT FUNGI ON BALSAM FIR SECTIONS

Fungi	Action
Daedalea unicolor	Fungus growth slow, little initial contact with wood, first bore holes through cut cell wall and pit membrane, later bore holes more numerous, through warty layer, no surface dissolution (Fig. 45)
Polyporus anceps	Fungus growth rapid, engulfs wood, frequent bore holes through the warty layer and pit immediately, initial bore holes 1 μm in diameter, may expand to 8 μm with longer treatment, no surface dissolution (Fig. 46)
Polyporus versicolor	Fungus growth fast, bore holes usually numerous, trails of partially dissolved warts associated with hyphal contact leads to surface dissolution (Fig. 47 and 48)
Poria subacida	Fungus growth fast, bore holes rare at first but more numerous with longer treatments, no surface dissolution
Schizophyllum commune	Moderate fungal growth, bore holes fairly numerous, 0.5-3.0 μm in diameter, initial bore holes usually through pit membranes, later more through warty layer, no surface dissolution (Fig. 44A and 44B)
Trametes suaveolens	Fungus growth very slow, little initial contact with wood, bore holes extremely rare, initial surface dissolution with warts partially dissolved in every tracheid without apparent hyphal contact (Fig. 49)

Action of Fungi

All six rots had at least some effect on the micromorphology of the cell wall. The attacks had two general forms: bore hole formation and surface dissolution. The localized action of bore hole formation allows the fungus to penetrate from one cell to another. All fungi investigated formed these

bore holes, but the frequency varied considerably. With Trametes, bore hole formation was extremely rare.

Figure 44A shows the tip of a branched hypha of Schizophyllum in the process of forming a bore hole. Figure 44B shows many bore holes through the cell wall caused by the same fungus after a longer incubation period. Bore holes are often formed first through the pit membranes which seemingly would be a path of less resistance (Fig. 45). It may be that the warty layer itself presents a temporary barrier to bore hole formation through the cell wall. When bore holes go through the entire cell wall, the warty layer must be dissolved before other cell wall constituents. The boring process is probably accomplished by enzymatic action localized at the tip of the advancing fungal hypha (128); however, the exact specificity of the enzyme(s) involved is not known. In Polyporus anceps, once the bore holes are formed, they can increase in diameter (Fig. 46), but this may occur by a different process than the initial formation.

The second type of attack of the fungi on the wood is surface dissolution, which may or may not involve intimate contact with the fungal hyphae. With white-rot fungi, cell wall dissolution proceeds from the lumen outward into the cell wall, and the first stage necessarily involves dissolution of the warty layer. Again, the specificity of the enzymes involved in this action is not known (128). This type of action was seen for both Polyporus versicolor and Trametes. Figure 47 shows a path of lytic action caused by Polyporus versicolor. Partial dissolution of the wart structure was caused probably by ectoenzymes secreted by the fungus (128). Figure 48 illustrates both localized bore hole formation and general cell wall dissolution by Polyporus versicolor. When cell wall dissolution is associated with fungal

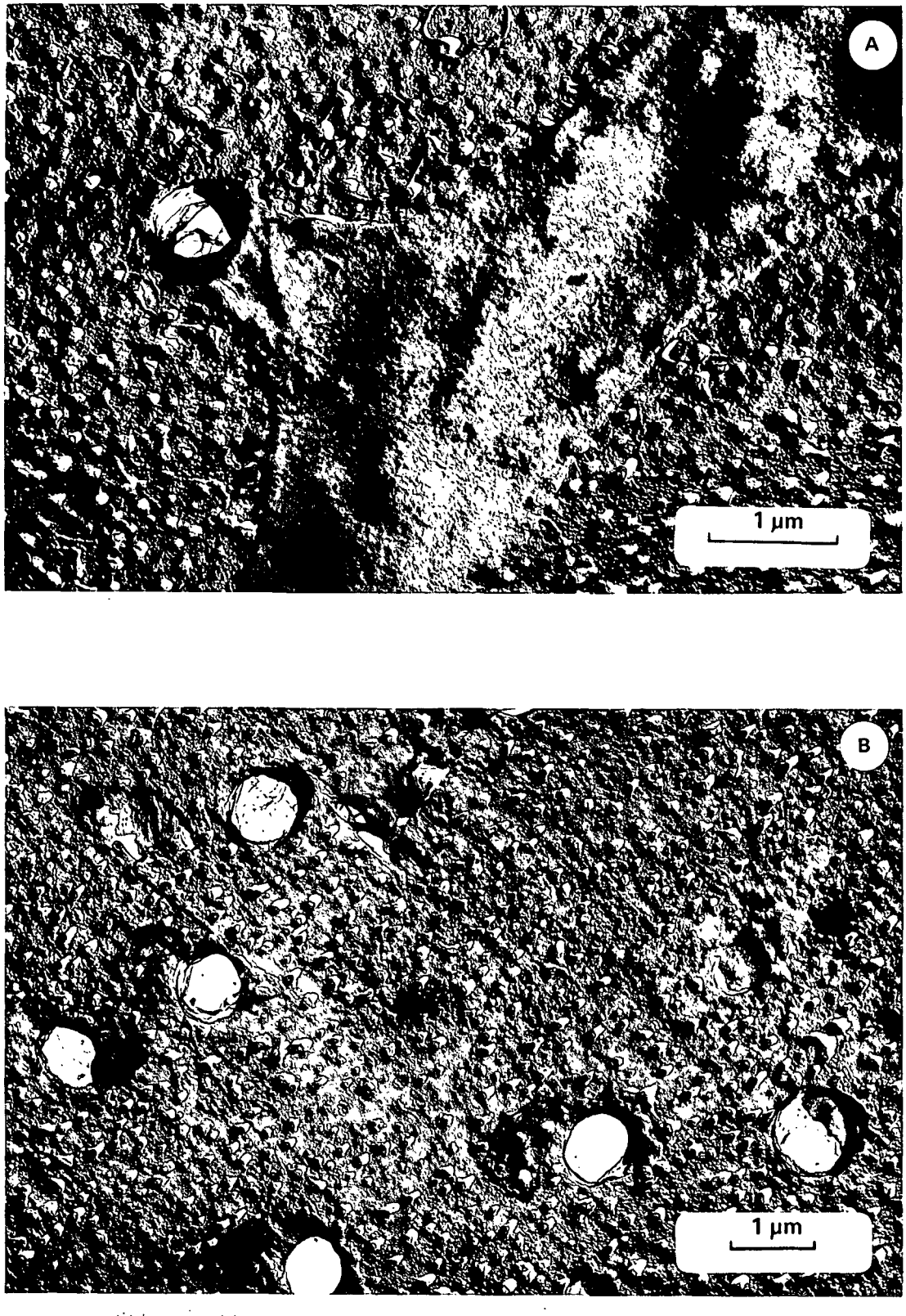


Figure 44A and 44B. Warty Layer Treated with Schizophyllum commune Fungus: A. 1 Week, B. 3 Weeks; Replicas, TEM Plates 10067 and 10068

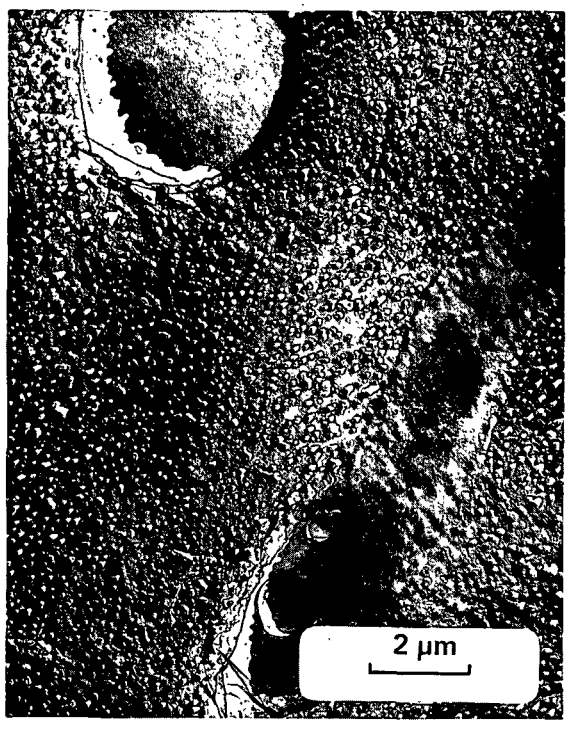


Figure 45. Warty Layer Treated with Daedalea unicolor Fungus for 3 Weeks; Replica, TEM Plate 10070

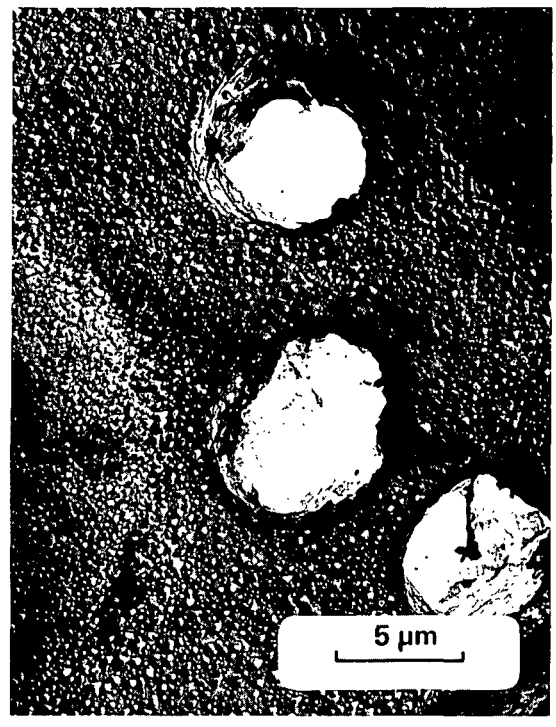


Figure 46. Warty Layer Treated with Polyporus anceps Fungus for 7 Weeks; Replica, TEM Plate 10126

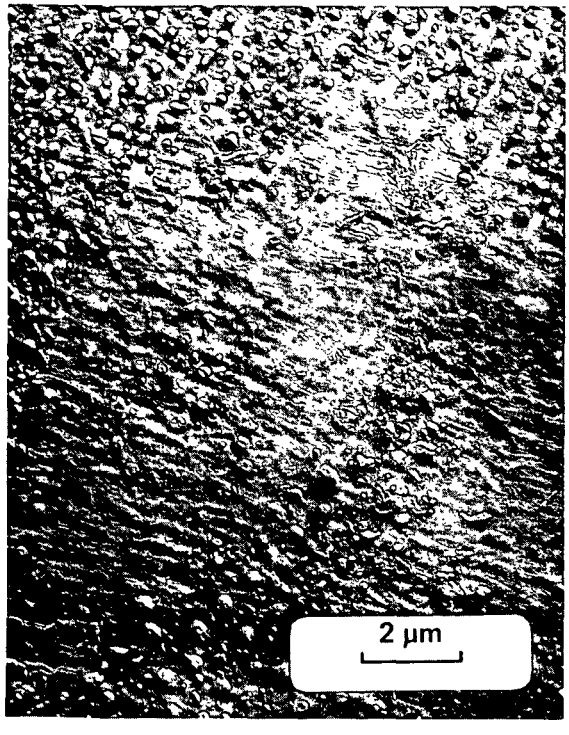


Figure 47. Warty Layer Treated with Polyporus versicolor Fungus for 10 Weeks; Replica, TEM Plate 10257

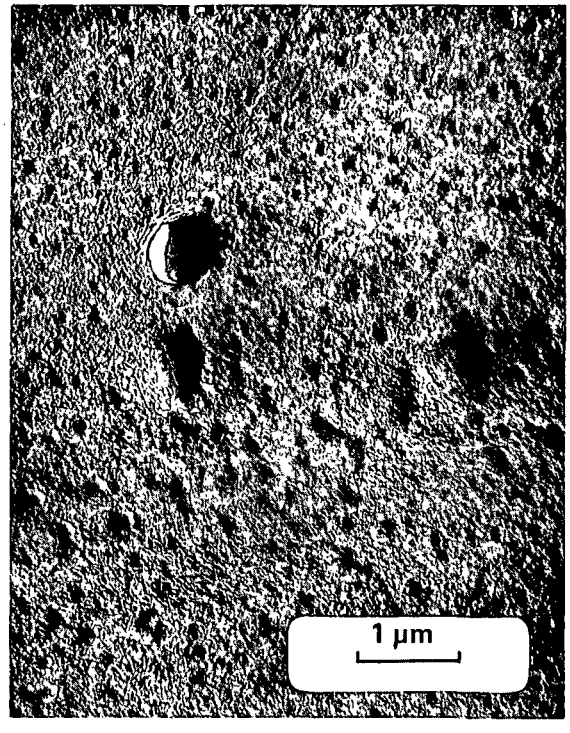


Figure 48. Warty Layer Treated with Polyporus versicolor Fungus for 10 Weeks; Replica, TEM Plate 10231

contact, the condition of the warty layer can vary from tracheid to tracheid, depending on the proximity of fungal hyphae.

Trametes was unique in its action in that it dissolved the warty layer from the lumen surface without apparent fungal contact (Fig. 49). The enzymes responsible for this action no doubt came from the fungus, but they apparently diffused throughout the nutrient medium and remained active. This was suspected since very few hyphae were seen along the cell wall, yet the dissolution of the warty layer was extensive in every lumen. Also, bore hole formation, which is necessary for migration of the fungus within the wood structure, was extremely rare.

The action of Trametes enzymes on the warty layer was similar to the action of H_2O_2. (Compare Fig. 49 and 38C.) Doughnut warts were also seen in the Trametes-treated fir as in the H_2O_2 treatment.

Since the Trametes took 3 to 5 weeks to develop a wart-dissolving ability, synthesis of the enzyme could have been induced by the presence of the wood substrate once some nutrient source in the medium was exhausted. There was no additional dissolution of the warty layer after the initial action even though not all of the structure was dissolved, indicating that perhaps the enzyme was active for only a limited time.

The wood exposed to Trametes culture for 8 weeks was subsequently treated with pectinase. The pectinase appeared to promote some continued dissolution of the warts, but it did not catalyze the removal of the amorphous layer (Fig. 50) as it did in fir holocellulose (see Fig. 43).

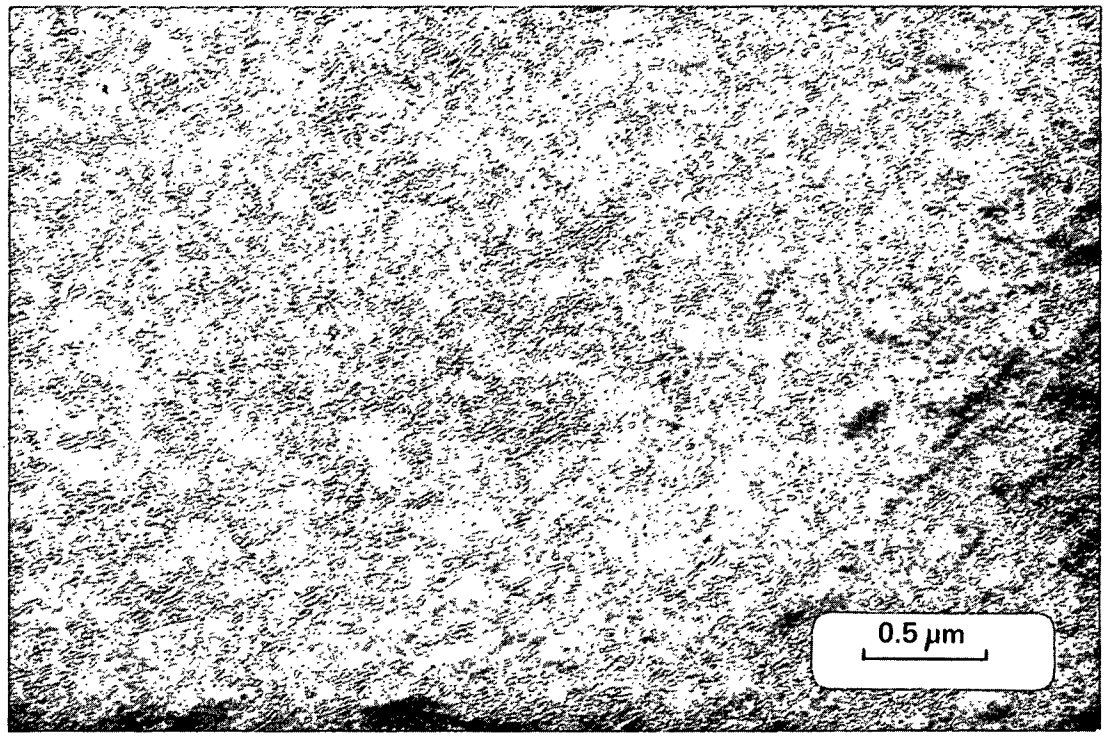


Figure 49. Warty Layer Treated with Trametes suaveolens Fungus for 8 Weeks; Replica, TEM Plate 10133

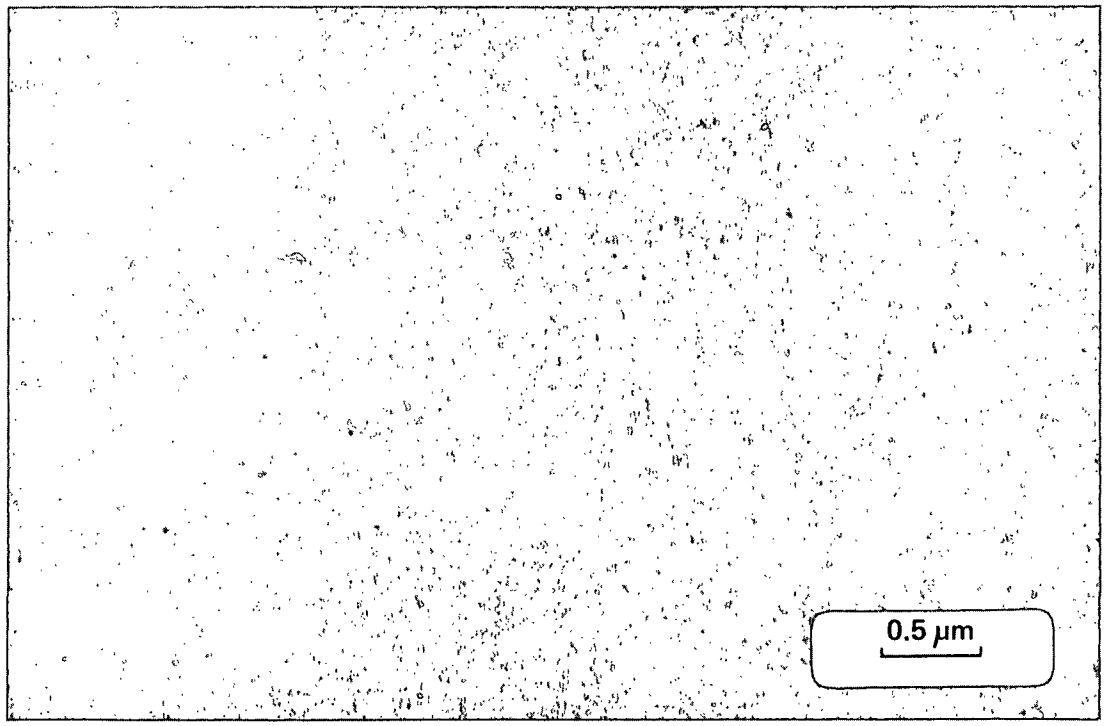


Figure 50. Warty Layer Treated with Trametes suaveolens Fungus for 8 Weeks, then Pectinase; Replica, TEM Plate 10135

Analysis of Trametes Extract

The medium was separated from the Trametes by centrifugation and ultrafiltration. The UV spectrum of the medium from a culture that had contained wood vs. the medium from a culture without wood had increasing absorbance at wavelengths decreasing from 340 nm. There were shoulders at 264, 286, 305, and 315 nm, which are in the range of lignin chromophores. A paper chromatographic analysis, however, failed to show any ligninlike monomers in the medium.

Fresh wood was introduced to the fungus-free culture medium, but no further enzyme activity was observed. The Trametes pellet from the centrifugation was resuspended in fresh asparagine-glucose nutrient. Unfortunately, the fungus had become contaminated with bacteria and was discarded.

Trametes from the stock source was again cultured in the presence of fir sections in an effort to repeat the enzymatic dissolution of the warty layer. Both asparagine-glucose and malt nutrient media were used. In none of five different cultures were the warts dissolved as extensively as in the original Trametes culture. The warts were partially dissolved in places, but they were more commonly unchanged. It is not known why the original results could not be repeated. Perhaps the enzyme system was active in the second set of tests but only for a very short period, producing only very limited attack on the warty layer. Otherwise, it is possible that the Trametes in the first culture underwent some mutation from the stock solution such that it gained a special wart-dissolving ability.

THERMAL TREATMENT

It is apparent that the warty layer undergoes thermal softening. When firwood was autoclaved at 121°C for 2½ hr, the warts showed signs of viscous

flow. They fell over from their natural protruding position and appeared to coalesce either with the amorphous layer or with other warts (Fig. 51). There was little noticeable effect with only ½ hr of this treatment.

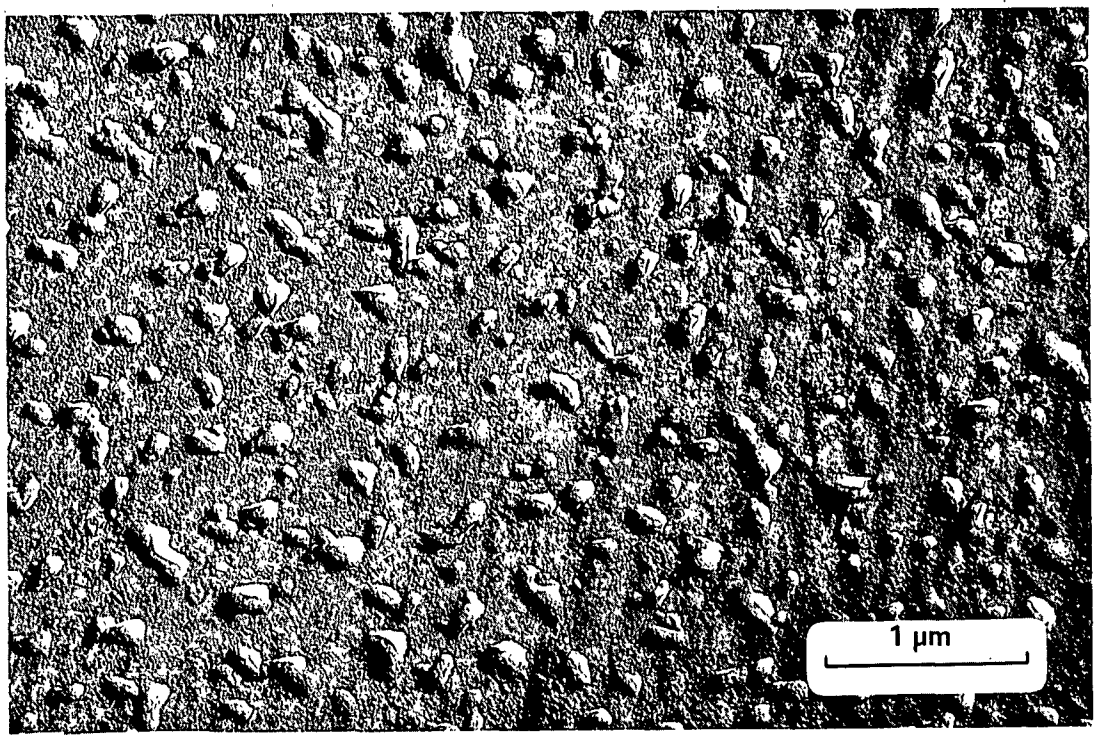


Figure 51. Warty Layer Treated with Steam at 121°C for 2½ hr; Replica, TEM Plate 10138

Goring (116) has determined the softening temperature for dry and moist isolated components of the cell wall. Of the moist samples, lignin softens at about 90-100°C, hemicellulose at 50-60°C, and cellulose at over 230°C. The behavior of the warty layer under autoclaving conditions is consistent with a composition of lignin or both lignin and hemicellulose.

Results of some of the other chemical-physical treatments probably also reflected thermal softening to some extent. During the first few hours of treatment with DMSO at 150°C, some of the warts could be seen lying down before any dissolution of the structure was apparent (Fig. 34A). With the alkali treatment, 0.1N KOH at 90°C, the warts also appeared to have melted-over to

coalesce with the amorphous layer (Fig. 28). Such action probably resulted from a combination of both thermal softening and reaction with the alkali, since there was no such effect on the wart structure when wood was treated with boiling water or with high alkali concentration at 60°C.

PHYSICAL ISOLATION

To chemically analyze the composition of the warty layer it would be most desirable to isolate it from the wood without chemical alteration. The basic obstacle here is that the warty layer is similar in its composition to other cell wall constituents. Dissolution of the warty layer, therefore, necessarily involves removal of other wood material, unless the warty layer can be isolated preferentially by virtue of its accessibility. This, however, did not prove to be the case for treatments investigated. Even when warts were removed, the treatments were never mild enough to ensure that the native chemical composition of the warts had been preserved. A successful method of physical isolation is apparently the only way to obtain uncontaminated, unaltered wart material. Three different procedures were attempted as described below, but none proved very successful.

Evaluation of Physical Isolation Techniques

Mechanical maceration of the wood and subsequent differential centrifugation up to 37,000 g for 15 min produced two distinct types of wood fragment in each fraction: numerous small angular pieces and some rodlike pieces. As was expected, higher rates of centrifugation sedimented smaller particle sizes, but the general particle shapes were similar in each fraction. At the highest centrifugation rates, the wood fragments had dimensions of 0.1 μm or less, but these particles resembled neither individual warts nor small fragments of the warty layer.

Ultrasonication of firwood in water, weak alkali, or pectinase did not free the warty layer from exposed tracheid lumens in either thin, transverse or thicker, radial sections.

The fragments isolated from scraped samples of polystyrene-embedded sections frequently included small patches of the amorphous layer with warts attached (Fig. 52). The isolated material also contained some microfibrillar substance. Such isolated fragments were, therefore, not pure warty layer components, but they were at least enriched with wart material.

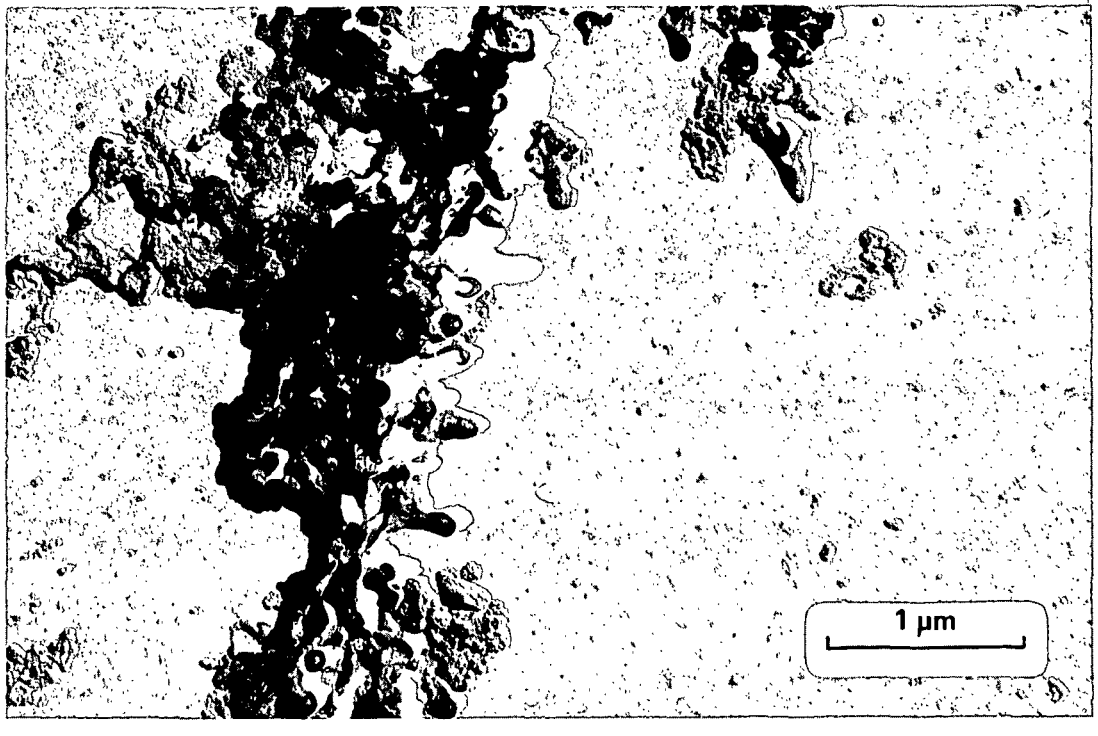


Figure 52. Warty Layer Isolated by Scraping Polystyrene-Embedded Wood Section; Replica, TEM Plate 9585

X-Ray Analysis

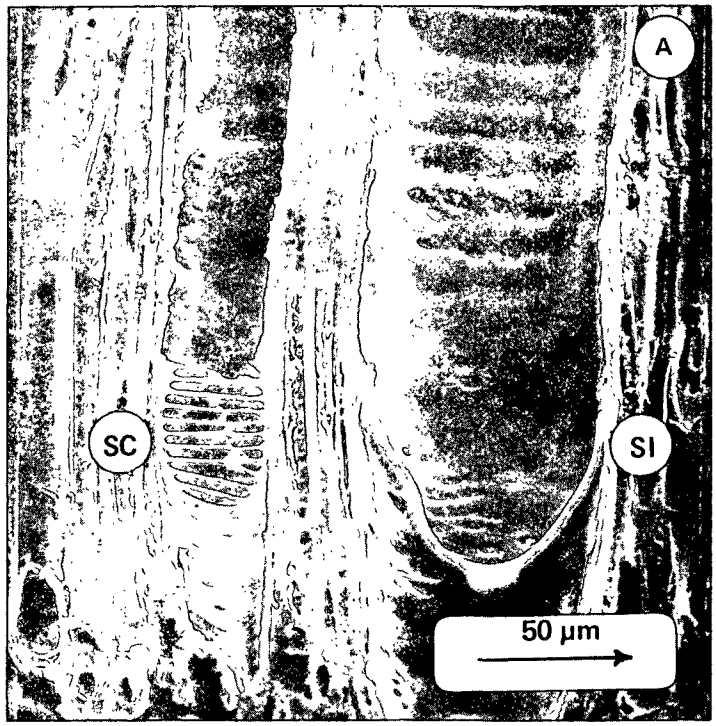
The yield from the polystyrene scraping was too small to permit chemical analyses. Also, since the material did not represent pure wart material, it would have been difficult to interpret the results of any such analysis.

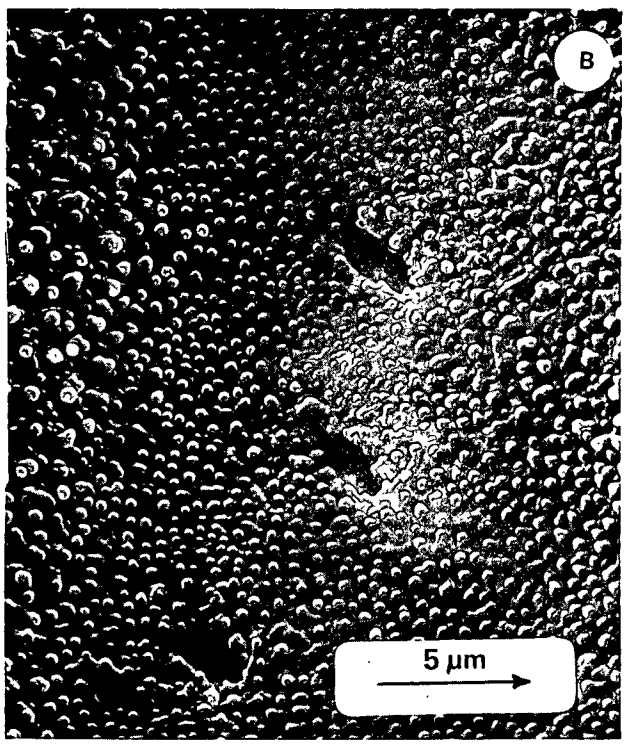
Specific areas, however, could be selected with the SEM and analyzed for elemental composition with the EDAX x-ray detection unit. The elements, calcium, potassium, silicon, chlorine, and occasionally sulfur and phosphorus were found in trace amounts in the wood fragments. The areas of the warty layer alone seemed to be richer in chlorine and potassium then other fragments of the cell wall. These elements function in the normal living cell to maintain osmotic and ionic equilibrium (131). It could be that they are transported across the cell membrane at the same time that the warts are formed. These elements are presumably combined in a water-insoluble form since the wood was saturated with distilled water during sectioning and storage. Young and Guinn (132), studying balsam fir trunkwood, reported finding most of the elements found here but no chlorine. Ellis (133), however, lists this element as a constituent of grand fir. He questioned the essentiality of chlorine but noted that it was always present.

TAXONOMIC SIGNIFICANCE OF THE WARTY LAYER

On examination of the wood morphology of American beech, a hardwood species, it was found that warts were present but occurred only in the smaller latewood vessel elements. Furthermore, all the wartless vessels had simple perforation plates, while warted vessels had either simple or scalariform perforation plates. Ohtani and Ishidi (41) recently reported a similar relationship in Japanese beech (Fagus crenata Bl.).

Figure 53A is an SEM micrograph of a radial section of American beech wood showing both a small and large vessel element with scalariform and simple perforation plates, respectively. Figure 53B is a high magnification view of the small vessel element showing the prominent warts on the lumen surface. Figure 53C is a similar view of the larger vessel element with a smooth surface.





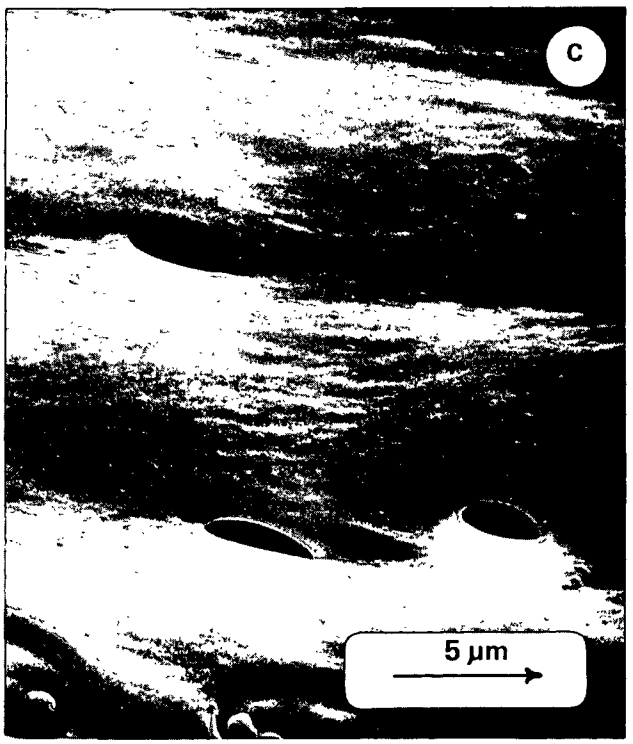


Figure 53A-53C. American Beech Vessel Elements: A. Scalariform (SC) and Simple (SI) Perforation Plates, B. Warty Layer of Vessel Element with Scalariform Perforation Plate, C. Smooth Surface of Simply Perforated Vessel; SEM Plates 634, 637, and 639

This relationship between the occurrence of warts and type of perforation plate in beech led to a study of other hardwood species that contain both simple and scalariform perforation plates.

It has been established that gymnosperms are the forerunners of angiosperms, which in an evolutionary sense have arisen comparatively recently (134). Hardwood cell types have, therefore, evolved from the conifer tracheid, and among the hardwood vessel elements, those with simple perforations are considered to be more advanced than those with scalariform perforations (134). Most wood species have exclusively either one perforation plate type or the other. Only a limited number of species exhibit both the more advanced and more primitive vessel types (32).

Fourteen species reported to have both plate types were examined, in order to determine if there is a correlation between cell morphology and the presence of warts and, therefore, a possible phylogenetic trend. This list included all of the North American species in this category listed by Panshin and de Zeeuw (77). Presented in Table IX are the results of the survey. In some of the species only one vessel perforation type was found (usually only simple). In each case the absent type was reported to be rare (77).

Within hardwoods containing both vessel elements with scalariform perforation plates (more primitive) and simple perforation plates (more advanced), warts, when present, were most often associated with only the former vessel type. Figure 54 depicts the warts on the scalariform perforation bars of sycamore. Figure 55 shows two small, warted vessel elements of sassafras also with scalariform end plates. Warts are always absent from the simply perforated vessel elements of sycamore and very rare in the simply perforated vessel elements of sassafras.

TABLE IX

OCCURRENCE OF THE WARTY LAYER IN ANGIOSPERM CELL TYPES

Species	Vessels		Fiber Elements
	Simple Perforation	Scalariform Perforation	
Corylaceae			
Carpinus caroliniana	No warts	--[a]	No warts
Ostrya virginiana	No warts	--	No warts
Cunoniaceae			
Ceratopetalum apetalum	No warts	No warts	No warts
Fagaceae			
Castanopsis chrysophylla	Very small warts, rare	No warts	Very small warts, common
Castanea dentata	No warts	--	No warts
Fagus grandifolia	Warts rare (Fig. 53C)	Always warted (Fig. 53B)	No warts
Fagus orientalis	Warts rare	Always warted	Small warts
Fagus sylvatica	No warts	Usually warted	No warts
Nothofagus moorei	No warts	Usually warted	No warts
Lauraceae			
Sassafras albidum	Warts very rare	Always warted (Fig. 55)	No warts
Magnoliaceae			
Magnolia acuminata	No warts	--	No warts
Magnolia grandiflora	--	No warts	No warts
Platanaceae			
Platanus occidentalis	No warts	Usually warted (Fig. 54)	Small warts
Ericaceae			
Oxydendrum arboreum	No warts	No warts	No warts

[a]This vessel type not observed in the samples examined.

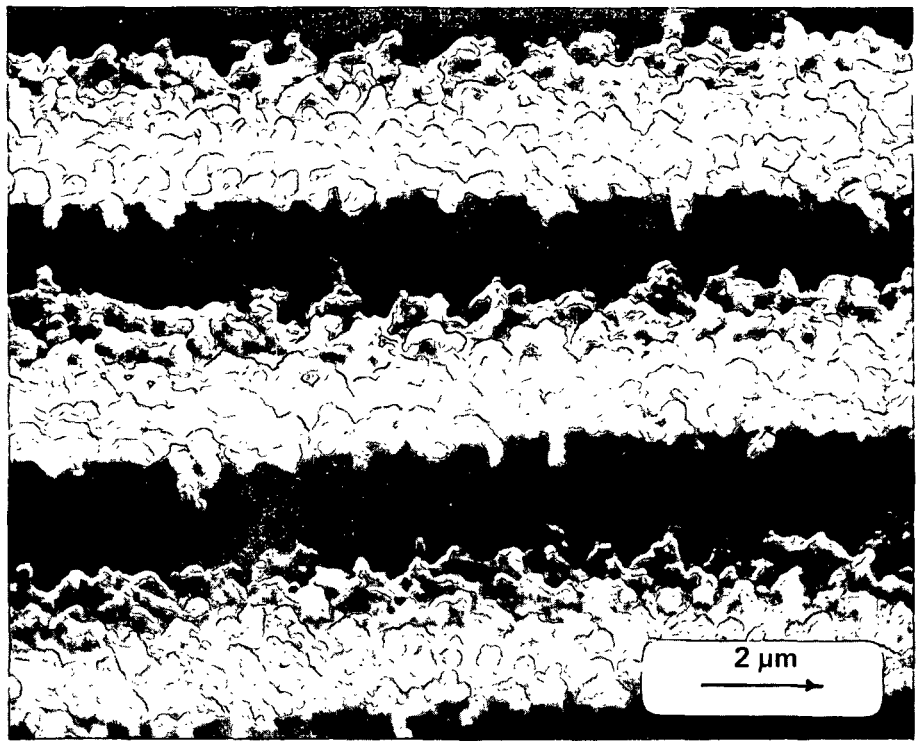


Figure 54. Warts on Scalariform Perforation Bars of American Sycamore; SEM Plate 654

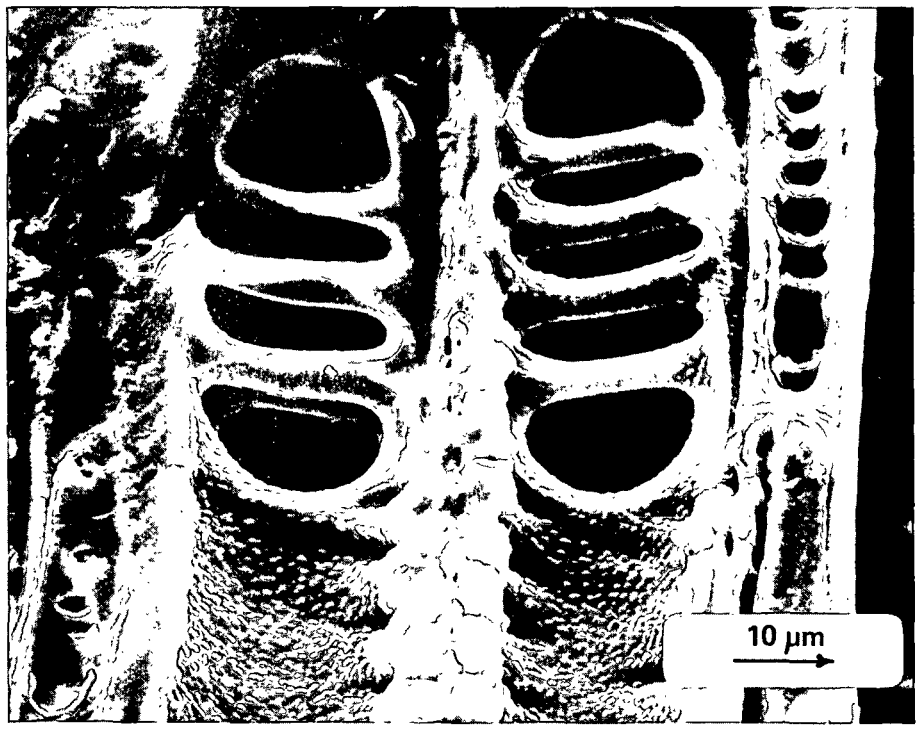


Figure 55. Warted Vessel Elements of Sassafras with Scalariform Perforation Plates; SEM Plate 715

In hardwood fibers, another highly specialized but imperforate cell type, only a few species displayed a warty layer, and then the wart structure was smaller than that found in the vessels. There was a complete absence of warts from the axial and ray parenchyma cells of all woods examined. No pit vestures were found in any of the species, though this structure has been associated with vessel warts (14, 37, 39).

A more complete discussion on the evolutionary significance of the presence of warts in angiosperm wood is presented elsewhere (34).

CONCLUSIONS

The warty layer in balsam fir is developed during the final stages of cell differentiation. After the microfibrils of the S3 layer have been deposited, the interior cell wall surface becomes slightly encrusted with an amorphous material and low mounds appear. The warty layer continues to develop until the S3 layer is completely covered with amorphous encrustant and the warts protrude into the lumen as blunt cones.

Most of the cell wall lignification process precedes wart formation. Judging from the permanganate staining reaction, when the developing warts are in the initial stage as low mounds, they are slightly more lignified than any portion of the adjoining secondary wall. In the final stage when warts protrude into the lumen, the warty layer appears much richer in lignin than the secondary wall. In a few cases, a two-component nature of the warty layer is observed where the outer portion is very darkly stained while the basal components of the individual warts is lighter.

Warts develop first at the tracheid corners and on the inner surface of the bordered pits and then on all walls over the whole length of the cell nearly simultaneously. This sequence in the wart formation pattern is analogous to the lignification pattern within the wall. The warts are formed exterior to the plasma membrane of the living cell before the cytoplasm has disappeared. No organelle or membrane other than the plasmalemma was found to have a specific association with wart formation, though this is not to say that no such association could exist. Also, no activity was observed within the cell wall directly beneath the point of wart formation. After warts are formed, the living cell contents degrade and flow from the tracheid, leaving no apparent, disorganized residue on the inner cell wall surface.

In conjunction with the study of developing tissue it was observed that the permeable margo region of the mature bordered pit arises from a gradual perforation of an initially solid membrane. The process occurs at the end of cell differentiation, after wart formation is complete, and is likely associated with the withdrawal of cytoplasm from the cell. Large, radially oriented microfibrils in the margo are present at an early stage of pit development when the membrane is still solid and do not arise as a result of aspiration of the mature pit.

From the response of the warty layer to many different treatments, it is concluded that the warty layer in balsam fir consists largely of a highly condensed ligninlike material. The interior, basal component of the individual warts and some of the encrusting layer consist of a noncellulosic carbohydrate, probably a pentosan or a pectic substance. Staining in cross section and direct surface examinations indicate that this basal component, and the slight S3 encrustant as well, are formed first by the cell and that the ligninlike covering is deposited later to complete the warty layer.

While the bulk of the warty layer is definitely ligninlike in reactivity, it is more resistant than, and thus different from, at least some of the other lignin in the cell wall. Depending on the treatment, from 16 to 65% of the lignin in wood was extracted before the warty layer, even though the warty layer was the cell wall component most accessible to the treatment solutions. From the results, it is concluded that the lignin in the warty layer is more concentrated and more condensed than the lignin in the rest of the cell wall.

In view of its chemical composition, it is not surprising that no chemical treatment was found that removed the warty layer exclusively. Analyses of the solutions in the different extraction series yielded little

additional information. Different chromatographic methods detected no unique monomeric unit associated exclusively with the lignin portion of the warty layer. The warty layer was extracted by some treatments as high molecular weight material confirming that this structure is probably of high molecular weight in the cell. No great change in UV absorbance or refractive index could be detected in the extraction solutions at the point that the warts were dissolved. There were changes in these latter properties as an extraction proceeded, but these changes were gradual over the entire treatment period and not just at the point where warts were extracted. Therefore, such changes could not be attributed to any specific cell wall structure.

The chemical reactivity of the warty layer, as well as that of other cell wall components, is altered by vacuum drying at 105°C. Under these conditions, irreversible dehydration and further condensation probably occurs to tighten the molecular structure, thereby diminishing accessibility and eliminating reactive sites. DMSO extraction is an exception, and the warty layer is somewhat more easily removed by this treatment after vacuum drying at 105°C.

The commercial enzymes used in this work had no effect on the mature, untreated warty layer. Different white-rot fungi attack the warty layer during localized bore hole formation or general cell wall dissolution. However, since the specificity of the enzymes involved is unknown, no conclusion can be drawn concerning the specific composition of the warty layer.

Thermal softening of the warty layer during steam heating is consistent behavior for the largely-lignin composition of the warts. Attempts at physical isolation of the warty component were not successful in producing material pure enough and in high enough yield for a definitive analysis.

From the results and conclusions on the development and composition of the warty layer, three hypotheses are offered to explain the formation of this structure in the tracheids of balsam fir:

1. Warts represent sites of material transport from the cytoplasm to the developing cell wall. When wall formation is complete, excess deposition causes the formation of the amorphous warts.

2. Warts are formed by deposition exterior to the plasmalemma of autolysis products of the dying cell with no relation to sites of previous deposition of cell wall components.

3. The wart structure is due to an eruption of material from the cell wall into the lumen caused by localized areas of high osmotic potential. These high pressure areas could develop as a result of a localized concentration of molecules exterior to the plasmalemma, possibly associated with cellulose microfibril synthesis and deposition or even the ends of small bundles of cellulose microfibrils. These areas of high osmotic potential act against the semipermeable cytoplasmic membrane, permitting wart formation.

The third hypothesis is intriguing and should not be discounted, but it is least satisfying of the three. Seemingly, any areas of high osmotic potential could be dissipated throughout the cell wall or at least evenly along the cell wall/plasmalemma interface, eliminating any localized eruption. Of the first two hypotheses, there are no results to present from this work or in work done elsewhere that would favor one over the other. Also, it is not unreasonable that some combination of the hypothesized processes operate to produce the total wart structure.

Some conclusions secondary to the main objectives of this work can be drawn regarding the significance of the wart structure, both in the processing of woody tissue and as a phylogenetic feature of wood. Balsam fir tracheids have a relatively heavy and resistant amorphous layer lining the lumen. Fir also was delignified with peracetic acid at a slower rate than loblolly pine, the tracheids of which have flat warts on an exposed S3 layer. The presence of a complete warty layer, therefore, may act as a barrier to the penetration of delignifying agents into the cell wall. It is risky to draw general conclusions from the study of the action of one delignifying reagent on only two species, but this explanation is certainly plausible and worthy of additional study.

In conjunction with this work, it was observed that the warts in mature loblolly pine are morphologically similar to the developing warts in fir. It is possible that the development of warts in pine follows the same initial stage as in fir but then stops.

In hardwood species that contain both vessels with scalariform perforation plates and vessels with simple perforation plates, the presence or absence of a warty layer follows a trend that can be associated with the degree of phylogenetic advancement of the xylem cell. The primitive-type vessels, those with scalariform perforation, are usually warted in those species that have warts. In the same species, the more advanced, simply perforated vessels only rarely exhibit warts. In hardwood fibers, another specialized but imperforate cell type, warts are also rare and very small when present.

GLOSSARY AND SPECIAL ABBREVIATIONS

Bordered Pit — intercellular opening with overhanging margins of the secondary wall and a membrane within the resulting chamber

Cambium — actively dividing layer of cells that lies between, and gives rise to, the xylem and phloem

Fusiform Initial — cambial initial that, through repeated division, gives rise to a radially directed row of longitudinal elements of xylem and phloem

Holocellulose — total carbohydrate fraction of wood remaining after the removal of lignin and substances extractable with neutral solvents

Lignin — random polymer of hydroxylated and methoxylated phenylpropanoid residues encrusting the cellulose framework of certain plant cell walls

Lumen — internal cavity of a wood cell formed when the living contents degrade and flow from the cell

Lumen Surface — lumen/cell wall interface; i.e., inner surface of the cell wall

Margo — peripheral region of the pit membrane lying between the torus and the border in bordered pits of softwoods

Pectic Substances — arabinans, galactans, and galacturonans

Phloem — inner bark tissue formed external to the cambium

Plasmalemma (plasma membrane) — unit membrane surrounding the cell cytoplasm

Ray Initial — cambial initial that gives rise to ray cells through repeated division

Ray Parenchyma — radially oriented, brick-shaped cells with generally simple pits

Scalariform Perforation Plate — perforated cell wall region consisting of multiple, parallel bars between longitudinally contiguous vessel elements in hardwoods

Simple Perforation Plate — cell wall region consisting of a single, large, round opening between longitudinally contiguous vessel elements in hardwoods

Tonoplast — unit cytoplasmic membrane enclosing a vacuole

Torus — central, thickened portion of the pit membrane in a bordered pit

Tracheid — longitudinally oriented, fibrous cell with bordered pits and imperforate ends

Warty Layer — amorphous structure containing numerous protuberances and covering the inner cell wall surface

Xylem — wood tissue inward from the cambium

dbh — diameter of tree at breast height

DMSO — dimethylsulfoxide, $(CH_3)_2SO$

EDAX — energy dispersive x-ray analyzer used with scanning electron microscope

FAG — chemical fixative with 2% formaldehyde, 2% acrolein, 3% glutaraldehyde, and 0.5% glucose in buffer

GAG — chemical fixative with 1% glutaraldehyde, 2.5% acrolein, and 0.5% glucose in buffer

S1,S2,S3 — outer, middle, and innermost layers, respectively, of the secondary wall of a wood cell

SEM — scanning electron microscope

TEM — transmission electron microscope

$\underline{t} \times \underline{r} \times \underline{l}$ — tangential by radial by longitudinal dimensions of a cut wood block

UTS — ultrathin section

SUGGESTIONS FOR FUTURE RESEARCH

The research presented here answers some old questions and poses some new ones. Several areas of future research are suggested by the results of this investigation.

It is hypothesized that the warty layer may be vestigial sites of material transport from the cytoplasm to the developing cell wall. If this is the case, one might expect to find a distinct pattern on the exterior surface of the plasmalemma that corresponds to the wart distribution. Applying freeze-etching techniques to differentiating cambial tissue could prove successful in exposing the exterior plasmalemma surface for examination with the electron microscope. An alternate approach might be to enzymatically dissolve the cell wall in the cambial zone to free individual protoplasts and permit examination of the membrane surface for any such pattern of material transport sites.

Autoradiography at the electron microscope level may prove useful in identifying radioactively-labeled precursors that eventually are incorporated into the warty layer. Both the development and the composition of the warty layer could be followed by this procedure.

No monomers uniquely associated with the warty layer were found by chromatographic analyses of the chemical extraction solutions. However, if the wart material is present in the extraction solutions only as a high molecular weight material, it may be necessary to first break the material down into monomers before analysis. This can be accomplished by several methods, including nitrobenzene oxidation to form characteristic aldehydes or ethanolysis to form so-called "Hibbert's ketones."

Further work could be done to identify the active agent(s) in the dioxane-HCl extraction of lignin from the wood.

It has been suggested in this work, without a great deal of documentation, that a chemically resistant warty layer may act as a barrier to the penetration of pulping liquors into the cell wall of certain species and thereby cause a decreased delignification rate. An experimental program could be developed to determine any relationship between the nature of the interior lining of the cell wall and the rate of liquid penetration into the wall.

Work could be carried out to follow up and confirm the EDAX x-ray analysis of warty layer fragments which tentatively showed that the warty layer was richer in chlorine and potassium than other cell wall components.

None of the commercially prepared enzymes used in this study had any apparent effect on the warty layer in mature wood. It is possible that with aging over several seasons the wart structure becomes more condensed and more resistant to attack. By using selected enzymes to treat a mature but only recently developed warty layer in the current annual increment, perhaps some specific action can be observed. The unknown enzyme activity from the white-rot fungi that dissolved the warty layer also could be investigated with respect to characterization.

The pectinase, which dissolved remnants of the warty layer from fir chlorite holocellulose, could be used to treat a similar residual structure in fir peracetic acid holocellulose to determine if the action is similar. A more specific polysaccharide-hydrolyzing enzyme than the pectinase used in this investigation could also be employed in an attempt to better specify the nature of the carbohydrate component of the warty layer.

Other interesting work to augment and extend the present investigation would be to study the development and composition of the warty layer in another softwood species, such as loblolly pine, in which the warts are structurally quite different than those in balsam fir. It would also be interesting to conduct a similar study of a hardwood species that contains warts in every vessel element or in a species, such as American beech, that contains warts only in some vessels. A study of the latter wood type might give some clues as to why some vessels develop warts (and often have scalariform perforation plates) while others have smooth cell wall linings (and simple perforation plates).

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APPENDIX I

CAMBIUM SAMPLING PROCEDURE

The outer bark was scraped away from about an 8 cm by 8 cm area of the tree trunk, 1½ m above ground level. Razor blade cuts were made down into the mature wood and a ⊏-shaped section of the tree was chiseled out as shown in Fig. 56.

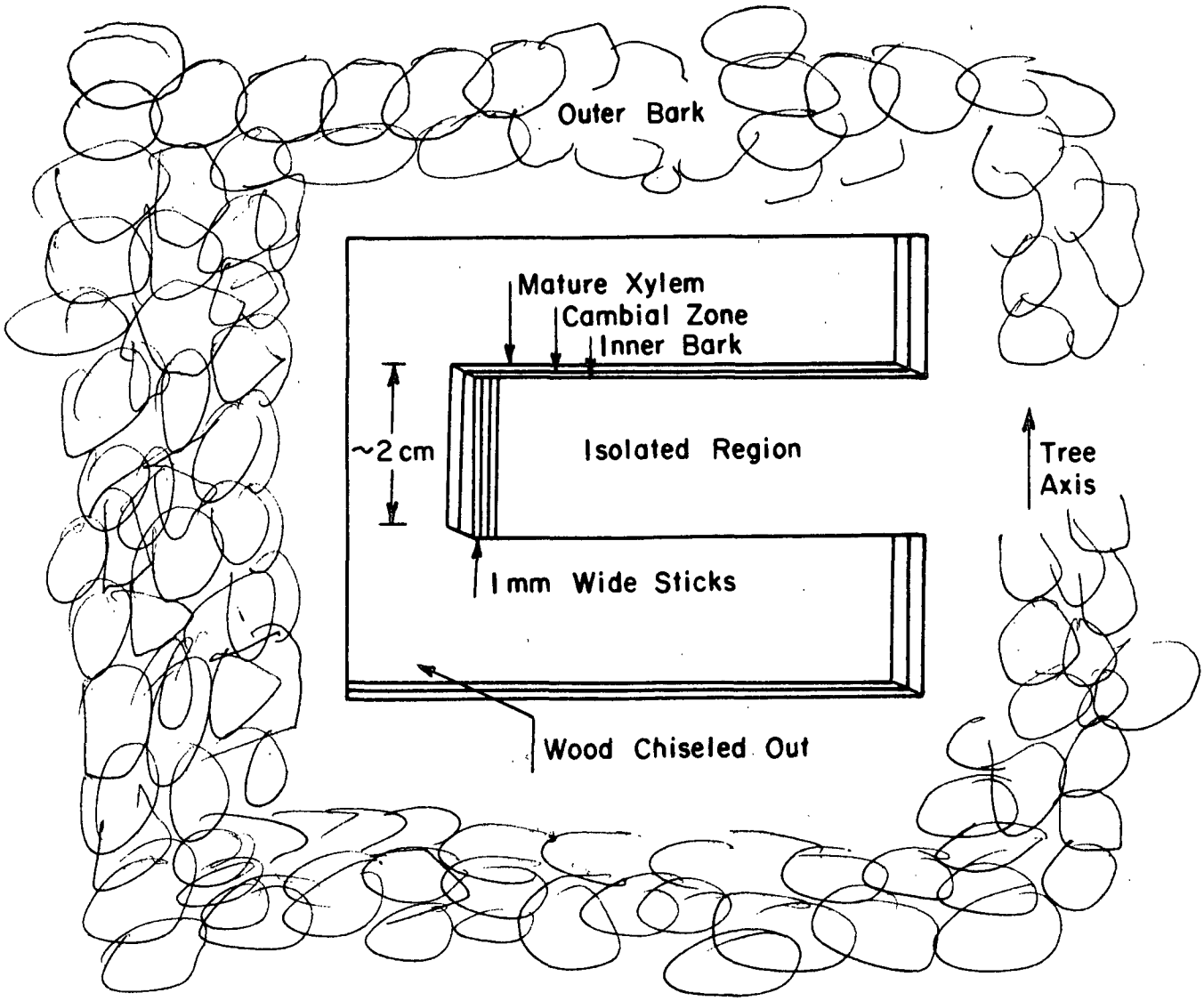


Figure 56. Diagram of Cambium Sampling Procedure

Vertical razor cuts 1 mm apart were made through the isolated region of the wood. The cuts were deep enough so that there was about 2 mm of phloem and 2 mm of xylem bordering the jellylike cambial layer. After 8 to 10 vertical cuts, resulting slices were carefully chiseled out in a group and placed immediately into the desired fixative solution (Appendix II). The procedure was repeated until the entire isolated region had been sliced and chiseled out.

Wider cambial samples (about 7 mm in the tangential direction) were also obtained in the same manner. These blocks were placed immediately in 30% ethanol for later collodion embedment (Appendix V).

After the cambial samples were removed, the cut areas were completely covered with rubber cement to prevent entry of any insect or pathogen. When more samples were needed, another 8 cm by 8 cm patch was cut above and to the side of the previous region and the procedure was repeated. Care was taken not to completely girdle the tree.

In the laboratory, the cambial slices were trimmed in Petri dishes while still in the primary fixative. About 2 mm was cut from each end and all but about 1 mm of the xylem and 1 mm of the phloem was trimmed away. Each slice was then cut transversely in half, leaving two slices about 1 mm × 2 mm × 5 mm ($\underline{t} \times \underline{r} \times \underline{l}$) containing the cambial tissue. The complete fixation schedule is outlined in Appendix II.

APPENDIX II

FIXATIVES

A. $KMnO_4$ fixative

1. Primary fixative — 2 hr, room temp., ±vacuum*

   2% $KMnO_4$, unbuffered

2. Rinse with water, dehydrate, embed (see Appendix III)

B. FAG fixative sequence (135)

1. Primary fixative — 3 hr, room temp., ±vacuum

   2% formaldehyde

   2% acrolein

   3% glutaraldehyde

   0.5% glucose

   all in 0.05M sodium cacodylate buffer, pH 7.2

2. Rinse with buffer

3. 1st Postfixative — 2 hr, 0°C

   2% $KMnO_4$ in buffer

4. Rinse with buffer, then water

5. 2nd Postfixative — 3 hr, 0°C

   0.5% uranyl acetate, unbuffered

6. Rinse with water, dehydrate, embed (see Appendix III)

C. GAG fixative sequence

1. Primary fixative — 3 hr, room temp., ±vacuum

   1% glutaraldehyde

   2.5% acrolein

*Vacuum applied several times intermittently to ensure penetration.

0.5% glucose

all in 0.05M sodium cacodylate buffer, pH 7.2

2. Rinse with buffer

3. 1st Postfixative — 3 hr, room temp.

   1% glutaraldehyde

   0.5% glucose

   all in buffer

4. Rinse with buffer

5. 2nd Postfixative — 12 hr, room temp.

   2% OsO_4 in buffer

6. Rinse with buffer, then water

7. 3rd Postfixative — 8 hr, room temp.

   aqueous saturated uranyl acetate, unbuffered

8. Rinse in water, dehydrate, embed (see Appendix III)

APPENDIX III

DEHYDRATION AND EMBEDMENT FOR ULTRATHIN SECTIONS

It is extremely important that all water is removed from tissue before embedment. Fixed cambial samples were, therefore, dehydrated according to the following sequence:

Solution	Minimum Time
15% Ethanol	15 min
30% "	15 min
50% "	15 min
70% "	15 min
90% "	15 min
95% "	15 min
Absolute "	5 min
" "	15 min
" "	Overnight
Propylene oxide	3 hr
" "	3 hr
" "	Overnight

A. Spurr embedment (136)

4.0 g DER 736 (diglycidyl ether of polypropylene glycol) for "hard" embedment

(6.0 g DER 736 for "firm" embedment)

10.0 g ERL 4206 (vinyl cyclohexene dioxide)

26.0 g NSA (nonenyl succinic anhydride)

0.4 g S-1 (dimethylaminoethanol)

The schedule for the infiltration of the fixed, dehydrated cambial samples with Spurr resin was as follows:

Propylene Oxide:Resin (v/v)	Time
1:1	4 hr
1:2	4 hr
0:1	Overnight
0:1	8 hr +vacuum

The resin was then polymerized at 65°C for 12 hr and then allowed to cool before sectioning.

B. Araldite 502 (137)

15.2 g Araldite 502 resin

11.3 g DDSA (dodecenylsuccinic anhydride)

0.375 g DMP-30 (2,4,6-trimethylaminomethyl phenol)

The procedure for infiltration and embedment of the fixed, dehydrated cambial samples with resin was as follows:

1. Place samples in Araldite:propylene oxide (1:1) for 12 hr, covered.
2. Uncover and allow to thicken to approximate consistency of pure resin (3 to 4 hr).
3. Transfer specimens to template, fill with undiluted Araldite, and allow to stand uncovered for 2 hr.
4. Cover and let stand at room temp. for 4 days, aspirating occasionally.
5. Complete polymerization at 60°C for 12 hr.
6. Cool for 12 hr before sectioning.

C. Epon-Araldite resin (138)

6.25 g Epon 812

3.75 g Araldite 502

13.75 g DDSA

0.75 g dibutylphthalate

0.5 g DMP-30 (add last)

mix thoroughly before use

The schedule for infiltration and embedment of the fixed, dehydrated cambial samples was as follows:

1. Propylene oxide:Epon-Araldite (2:1), 4 hr.
2. Propylene oxide:Epon-Araldite (1:2), 4 hr.
3. Epon-Araldite, overnight.
4. Epon-Araldite, 4 hr.
5. Transfer wood slices to filter paper for draining, then to fresh Epon-Araldite in embedding mold. Aspirate to remove air bubbles.
6. Polymerize at 60°C overnight, and allow to cool before sectioning.

APPENDIX IV

STAINING PROCEDURES

After the fixed and embedded cambial samples had been sectioned with the ultramicrotome and placed on grids, they were stained by one or both of the following procedures:

A. 0.5% Lead citrate, pH 12.

1. Put drops of stain onto a wax bottom Petri dish with a few pellets of KOH nearby to reduce CO_2 contamination. Avoid breathing on drops.

2. Float grids on the drops, section side down, for 10 to 20 min in the covered dish.

3. Rinse successively in 0.02N NaOH and then three vessels of distilled water with 20 rapid dips into each vessel.

B. 2% Potassium permanganate.

1. Pipet stain from under solution surface and drop onto wax bottom Petri dish.

2. Float grids on the stain, section side down, for 10 to 20 min in the covered dish.

3. Rinse in three successive vessels of distilled water, 20 rapid dips each.

After staining and rinsing, grids were dried by blotting the very edge with lens paper. The water was thereby drawn off by capillary action.

APPENDIX V

COLLODION EMBEDMENT FOR MICROTOME SECTIONING (139)

Cambial sample blocks which included about 2 mm of tissue on either side of the cambium band were dehydrated and embedded in collodion for subsequent sectioning. The samples were trimmed to about 1 cm × 0.5 cm × 1 cm (t × r × l) before the following dehydration and embedding schedule:

Solution	Time
30% Ethanol	2 hr
50% "	1 hr
70% "	1½ hr
85% "	1½ hr
90% "	2 hr
Absolute "	2 hr
" "	Overnight
" "	1 hr
Ethanol:ether (1:1)	4 hr
2% Collodion*, 100 psi	8 hr
2% Collodion, atm. pressure	1 week
12% Collodion, 100 psi	8 hr
12% Collodion, atm. pressure	1 week

The collodion-embedded block was then hardened and sectioned as follows:

1. Thicken collodion to a heavy syrup by evaporation of solvent from uncovered vessel.
2. Remove wood samples and plunge them into about 10 volumes of chloroform for 12 hr to harden the collodion.
3. Section the embedded wood radially with a sliding microtome at about 100 µm.
4. Dissolve collodion from the sections using ethanol:ether (1:1) with three 1-hr changes.
5. Dry sections from ether.
6. Prepare sections for electron microscopy.

*Collodion solutions were prepared with Parlodion (a highly purified cellulose nitrate produced by Mallinckrodt) dissolved in absolute ethanol:ether (1:1).

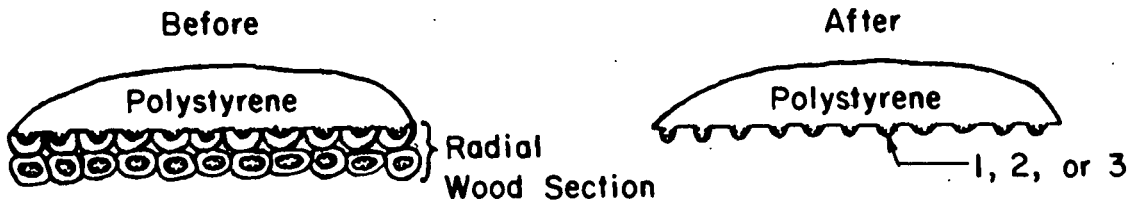
APPENDIX VI

POLYSTYRENE BACKING

Wood sections were backed with molten polystyrene with the apparatus described by Dunning (43). The technique was used in this investigation for three different purposes:

1. The original purpose was to give support to replicated surfaces while the wood was being dissolved.
2. Wood sections were backed during the harsh 72% H_2SO_4 treatment to maintain the organization of any cell wall features not dissolved.
3. In an attempted physical isolation of the warty layer, wood sections were backed with molten polystyrene, and all of the wood was then carefully scraped away with a dissecting needle, hopefully leaving only the minute warts from the interior cell wall surface embedded in the polystyrene.

Schematically, these situations should exist before and after treatment:



Cross section view of polystyrene backed wood section

Polystyrene, plus:
1) carbon replica after dissolution of wood,
or 2) residue left after H_2SO_4 treatment,
or 3) embedded fragments left after wood scraped away.
Numbers correspond to the procedures listed above

APPENDIX VII

CULTURE MEDIA

The following media were used as nutrient solutions for the culture of white-rot fungi:

A. Asparagine-glucose medium (140)

Glucose	20.0 g	Citric acid	1.4 mg
L-asparagine	2.0 g	$FeSO_4 \cdot 7H_2O$	1.0 mg
KH_2PO_4	3.0 g	Thiamine·HCl	1.0 mg
$MgSO_4 \cdot 7H_2O$	0.5 g	$CuSO_4 \cdot 5H_2O$	0.4 mg
$CaCl_2$	3.4 mg	$MnSO_4 \cdot H_2O$	0.3 mg
$ZnSO_4 \cdot 7H_2O$	1.8 mg	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.3 mg

bring to 1 liter with distilled water

B. Malt medium

1.5% Malt extract (product of Difco Laboratories) in water.

APPENDIX VIII

DEVELOPING SOLVENTS AND DETECTION REAGENTS FOR THIN-LAYER AND PAPER CHROMATOGRAPHY

Spotted chromatograms were developed with one of the following solvent systems:

Solvent I — Ethyl acetate-isopropanol-water (8:2:1). This solvent was used to fractionate sugars on thin-layer plates.

Solvent II — Ethyl acetate-acetic acid-formic acid-water (18:3:1:4). This solvent was used to fractionate sugars on paper.

Solvent III — Ethyl acetate-pyridine-water (8:2:1). This solvent was also used to fractionate sugars on paper.

Solvent IV — Butanol-pyridine-water (10:3:3). This solvent was used to fractionate lignin monomers on paper.

All solvent ratios were based on volume at room temperature.

Developed chromatograms were allowed to dry and were then detected by spray reagent.

I. Carbohydrate detection reagents

A. Anisaldehyde reagent (141)

0.5 ml anisaldehyde

0.5 ml concd. H_2SO_4

0.1 ml glacial acetic acid

9.0 ml 90% ethanol

Chromatograms were sprayed with a freshly prepared solution, air dried, and heated at 100°C for 5 min. Different monosaccharides gave distinguishing color reactions (142).

B. p-Anisidine-hydrochloride (143)

Mix in the following order:

0.5 g p-anisidine hydrochloride

5 ml water

10 ml 95% ethanol

85 ml n-butanol

Chromatograms were sprayed, air dried, and heated at 105°C for 5 min. The pentoses and uronic acids appeared pink to red and the hexoses appeared brown.

II. Lignin detection reagents

A. 2,4-Dinitrophenylhydrazine (127)

Chromatograms were sprayed with a saturated solution of 2,4-nitrophenylhydrazine in 2N HCl and air dried. Carbonyl groups were detected as orange or brown spots on a light yellow background.

B. Diazotized p-nitroaniline (120)

After drying, the developed chromatogram was exposed to ammonia vapor and sprayed immediately with a 0.05% solution of the diazo salt of p-nitroaniline in water. After air drying and recording of any color spots, the chromatogram was sprayed with a saturated solution of sodium carbonate in water and allowed to air dry. Phenolic compounds and aromatic amines gave characteristic colors (120).