

AN ABSTRACT OF THE THESIS OF

John Dennis Litvay for the degree of Doctor of Philosophy
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Title: ANATOMICAL AND CHEMICAL CHARACTERISTICS OF THE
DOUGLAS-FIR (PSEUDOTSUGA MENZIESII (MIRB.) FRANCO)
PHELLEM CELL

Signature redacted for privacy.

Abstract approved: _____

Robert Krahmer

The objectives of this study were to 1) characterize the layering in the wall of the Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) phellem cell, 2) determine the pitting present in this cell wall and 3) determine what compounds are present in the middle lamella of this cell.

To accomplish the objectives excised Douglas-fir cork was subjected to various chemical treatments (extraction with organic solvents, water, ammonium oxalate and sodium sulfite, saponification by an aqueous sodium hydroxide solution and an ethanolic potassium hydroxide solution). At certain stages in the treatment samples were removed for such analyses as trace mineral, protein and specific gravity. Paralleling this microtomed sections of cork were similarly chemically treated and removed for observation with various types of microscopes. The microscopes used were light,

ultraviolet, polarizing, scanning electron and transmission electron.

The results of this study showed that the cell wall structure of the Douglas-fir phellem cell is composed of 4 zones or layers. They are 1) a primary wall with randomly oriented microfibrils, 2) a secondary suberin layer composed of alternating lamella of phenolics and waxes which have the wax molecules oriented perpendicular to the cell surface, 3) a wax extractives layer and 4) a layer of cellular debris such as cytoplasm and old membranes lining the lumen. A middle lamella composed of phenolic and pectic substances holds the cells together.

The simple pits of the phellem cell are plugged with a material which gives a callose reaction when stained with aniline blue.

Also the first formed tangential wall of the first formed uncrushed phellem cell in a growth increment has an abnormally thick compound middle lamella.

Anatomical and Chemical Characteristics of the
Douglas-fir (Pseudotsuga menziesii (Mirb.)
Franco) Phellem Cell

by

John Dennis Litvay

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Date thesis is presented

December 12, 1975

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Christmas will soon be here and I hope I may be with those who have been most helpful to me, my family. My mother, father, aunts, uncles and in-laws who have all helped me; especially to my wife Darlene who has given of herself, unselfishly throughout the years.

TABLE OF CONTENTS

INTRODUCTION	1
PREVIOUS WORK	3
DESIGN OF EXPERIMENT	4
RESULTS AND OBSERVATIONS	18
Effect of Time, Alkali and Solvent in the Saponification Reaction	18
Chemistry	28
Yield Data for the Extractions and Chemical Treatments	28
Analysis of the Water Soluble Fraction of the Benzene-Ethanol Extractives	30
Analysis of Alkali Saponifiables	32
Protein and Trace Mineral	34
Acid Hydrolyzates	37
Specific Gravity by Liquid Immersion	37
Anatomy	43
Polarization	43
Ultra-Violet	47
Scanning Electron Microscopy	51
Transmission Electron Microscopy	60
DISCUSSION	84
CONCLUSIONS	98
EXPERIMENTAL	99
Chemistry	99
Collection and Preparation of Material	99
Chemical Treatment of Cork	99
Analysis of the Water Solubles from the Benzene-Ethanol Extractives	103
Analysis of Saponification Solubles	103
Crude Protein Determination - Micro Kjeldahl Method	103
Total Ash	108
Basic Method for Detection of Trace Minerals by Atomic Absorption	108
Specific Gravity Determination by Liquid Displacement	109
Strong Acid Hydrolysis	110

Anatomy	111
Collection of Sample Material Used for Sections	111
Chemical Treatment	112
Polarizing Microscopy	112
Ultra-Violet Microscopy	112
Scanning Electron Microscopy	113
Transmission Electron Microscopy	114

BIBLIOGRAPHY	120
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LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Cross section of a mature Douglas-fir stem	4
2. Douglas-fir bark	4
3. Cork cell types found in Douglas-fir bark	6
4. Growth increments of cork within a periderm	6
5. Proposed models of cork cell walls	8
6. Procedure used to study the parameters involved in the saponification reaction	15
7. Chemical treatments given to Douglas-fir cork	16
8. Effect of time in the saponification reaction	19
9. Effect of alkali in the saponification reaction	23
10. Effect of solvent in the saponification reaction	26
11. Chemical components present (by percent) in the Douglas-fir phellem cell	29
12. Results of protein and trace mineral analysis	36
13. Paper chromatographs of the acid hydrolyzates for detection of sugars	38
14. Diagram representing the double wall between two phellem cells	41
15. Polarization pictures of Douglas-fir cork having various chemical treatments	44
16. Polarization picture of macerated cork and diagrams representing the cell in 16A	46
17. Pictures of Douglas-fir cork having various chemical treatments, taken with an ultra-violet light source	49

<u>Figure</u>		<u>Page</u>
18.	Scanning electron micrographs of the ground cork used in the chemical analysis	52
19.	Scanning electron micrographs of cork sections having various chemical treatments	55
20.	Transmission electron micrographs of cork replicas, representing various chemical treatments	61
21.	Transmission electron micrographs of unextracted cork sections showing differences in staining techniques	66
22.	Transmission electron micrographs of cross and radial sections of cork stained with osmium after an acid pretreatment	71
23.	Transmission electron micrographs of radial sections of cork stained with osmium after acid pretreatment showing the effect of various chemical treatments on the cell wall	72
24.	Transmission electron micrographs of radial sections of cork showing variations in wall thickness	77
25.	Transmission electron micrographs of radial sections of cork showing the existence of callose and the type of pitting present in the cork cell wall	79
26.	Transmission electron micrographs of the ground cork used for chemical analysis	82
27.	Proposed model for the cell wall of a Douglas-fir phellem cell	85
28.	Untreated cork sections showing deposits of tannins, dihydroquercetin and other extractives	89
29.	Possible orientation of molecules in a wax lamella in the suberin layer using palmitic acid and ferulic acid as model compounds	92
30.	Procedure used in the analysis of the water soluble fraction of the benzene-ethanol extractives	104

<u>Figure</u>		<u>Page</u>
31.	Procedure used in the analysis of the aqueous sodium hydroxide saponification reaction solubles	105
32.	Procedure used in the analysis of the ethanolic potassium hydroxide saponification reaction solubles	106
33.	Orientations and cutting patterns used in the embedment of cork sections for transmission electron microscopy	116
34.	Steps used in the preparation of the replicas used for transmission electron microscopy analysis	118

LIST OF TABLES

<u>Table</u>		<u>Page</u>
1.	Comparison of suberin contents found by various investigators on various species of cork	12
2.	Analysis of alkali saponificables according to pH and acetone solubility	33
3.	Specific gravity of cork cell wall by the liquid immersion technique using four different liquids	40
4.	Values for the thickness of layers found in the cork cell walls of <u>Quercus suber</u> and <u>Pseudotsuga menziesii</u> (Mirb.) Franco	86
5.	Acid hydrolysis calculations	111

ANATOMICAL AND CHEMICAL CHARACTERISTICS OF THE
DOUGLAS-FIR (PSEUDOTSUGA MENZIESII (MIRB.)
FRANCO) PHELLEM CELL

INTRODUCTION

Considerable quantities of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) bark are accumulated each year in the Pacific Northwest as a result of manufacturing activities in the forest products industries. Cork is a major component of this bark, ranging from 30-50% of the dry bark weight (23).

Cork has been shown to have different physical properties than wood or bark fiber (26, 27). Also, cork imparts particular properties to products made from bark. For example, irregular surfaces may occur on bark hardboards because the spongy cork cells pop back after being compressed in the hot press. Also, hardboards made without adhesives from Douglas-fir bark particles have the highest internal bond strength when cork is present because of thermoplastic flow and physical bonding of cork particles (42).

Despite this knowledge of the properties of Douglas-fir cork, very little is known about the cells that are present in cork. The goal of this study is to learn more about the major component of cork, which is the phellem cell, and therefore give some predictability for the observed properties of cork.

Specifically the objectives are: 1) characterize the layering

in the wall of the phellem cell, with respect to the number of layers and chemical makeup; 2) determine the type of pitting present in this cell; and 3) since lignin has not been considered to be present in cork cells, determine what compound or compounds are present in the middle lamella between phellem cells.

PREVIOUS WORK

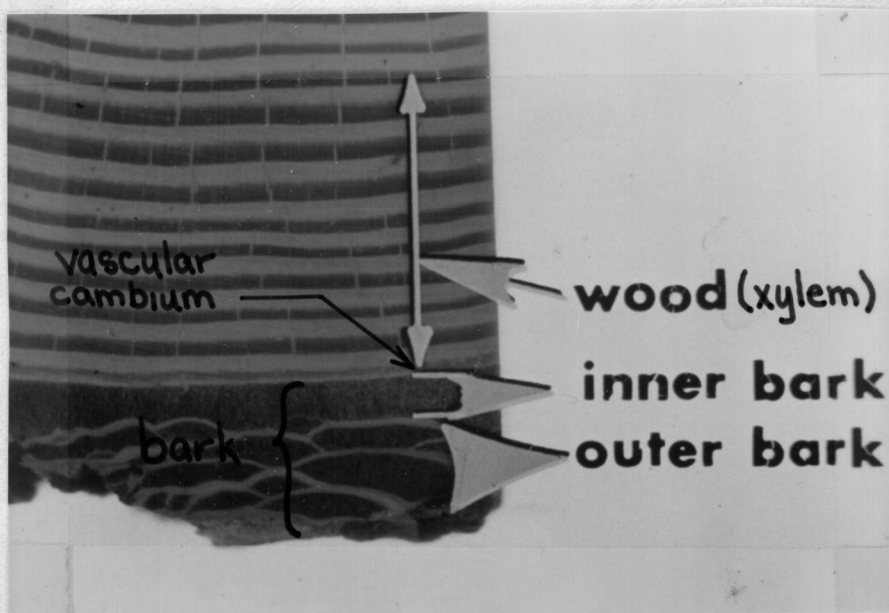
A cross section of a mature Douglas-fir stem reveals two zones, wood (xylem) and bark (Figure 1). Bark will be defined as all tissues external to the vascular cambium.

The bark on the mature stem can similarly be divided into two regions, the inner bark (phloem) and the outer bark (rhytidome), which are separated by the last formed cork layer. This cork layer also contains a meristamatic tissue region called the cork cambium. New cork cambia periodically form in the inner bark presumably from phloem parenchyma and ray parenchyma (13). In so doing, a portion of the inner bark is cut off and is now considered to be part of the outer bark. Thus the outer bark is composed of alternating layers of cork and phloem tissue (Figure 2). When phloem tissue is cut off from the inner bark, the tissue dies because no food stuffs are conducted through the cork layer.

A single cork layer is called a periderm. The time and position at which periderm formation takes place differ among tree species. Periderm thickness in Douglas-fir generally does not exceed $1/2''$, although very thick periderm has been observed particularly near the root collar (23, 33). A good bibliography on periderm formation and factors influencing periderm formation can be found in Borger (3).

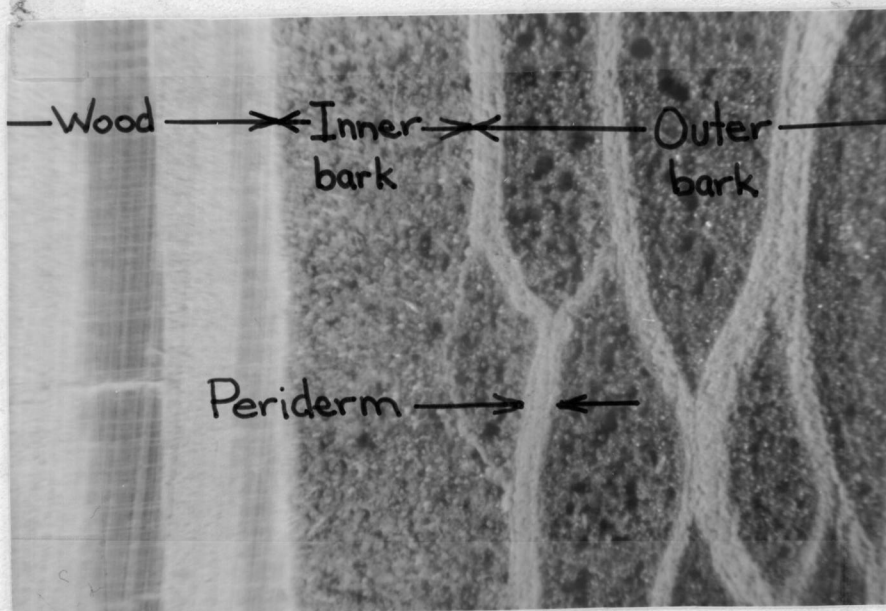
A periderm is composed of three morphological tissues, the

Figure 1. Cross section of a mature Douglas-fir stem



mag. 3X

Figure 2. Douglas-fir bark



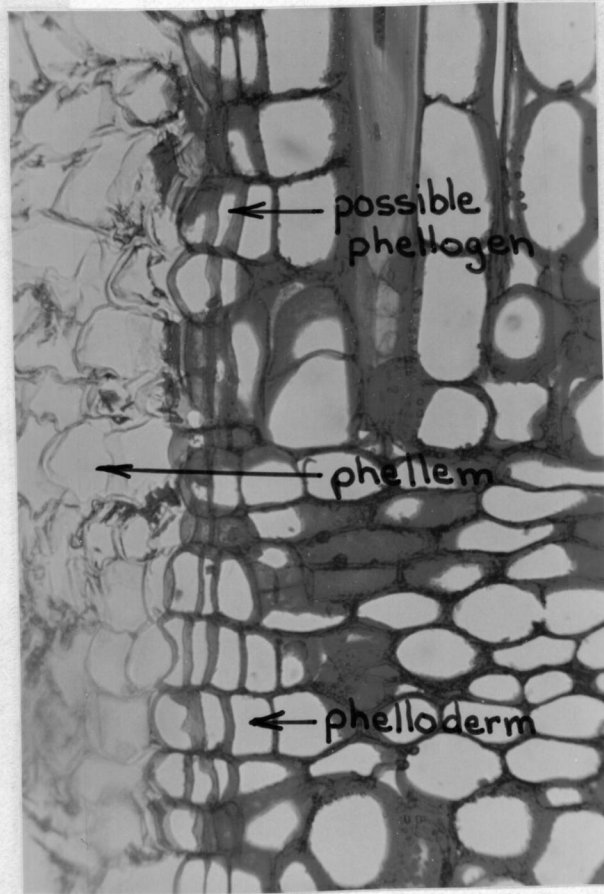
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phellogen (cork cambium), the phlelem (newly formed phellum cells which lie to the outer bark side of the cork cambium) and the phel-loderm (newly formed cork cells which lie toward the inner bark side of the cork cambium and are only 2 to 3 cells wide regardless of the thickness of the periderm) (Figure 3). All cells in the periderm layer are aligned in well oriented radial rows; however, the phellem cell forms the majority of the layer and will be the only cell type described in this study.

In Douglas-fir, growth increments consisting entirely of phellem cells occur within a periderm at fairly regular intervals (23) (Figure 4). Grondal (14) has called these "annual increments," although the time period for an increment to develop has not been established. According to Krahmer and Wellons (23) the growth increments are the result of bands of light colored, uncrushed cells which alternate with bands of darker severely crushed cells. The severe folding and crushing of the radial cell walls results in an increase in bulk density. When this crushing occurs is also unknown, but it is thought to take place after full development of all cells in the increment (23). Also, Krahmer and Wellons (23) have noted that the amount of radial compression is quite similar from increment to increment within an individual cork layer, and even to a large extent in all cork layers from approximately the same height in an individual tree, and that the greatest variation in compression appears to occur from tree to tree.

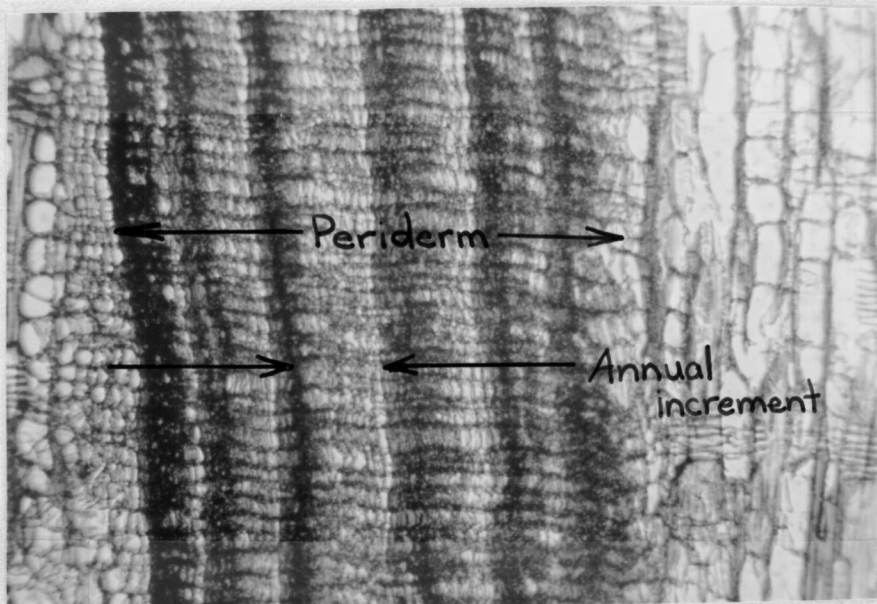
Figure 3. Cork cell types found in Douglas-fir bark

6



mag. 163X

Figure 4. Growth increments of cork within a periderm



mag. 53X

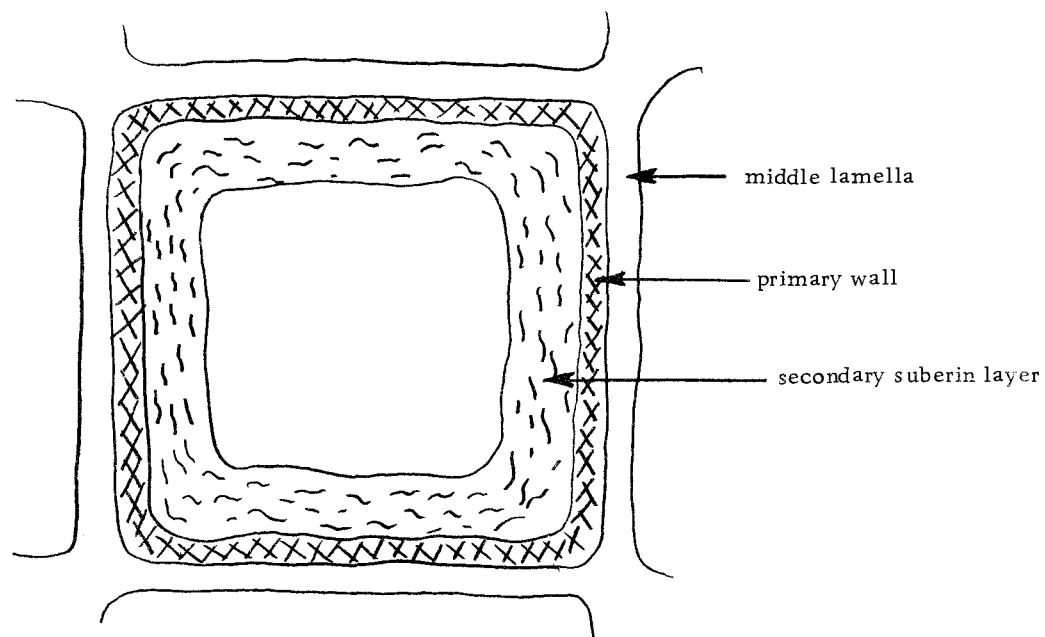
Also the dark color in some cells of the increment is thought to be due to heavier deposition of extractives in the lumen of the phellem cell.

An individual phellem cork cell is somewhat isodiametric and symmetrical in shape. The radial dimension is about 30 micrometers, tangential about 50 micrometers, and they are about 60 micrometers long. Their shape in transverse and radial sections is square to rectangular. On the tangential surface most of the cells are irregularly pentagonal to hexagonal in shape and are arranged in such a manner as to have no intercellular space between adjacent cell walls (23).

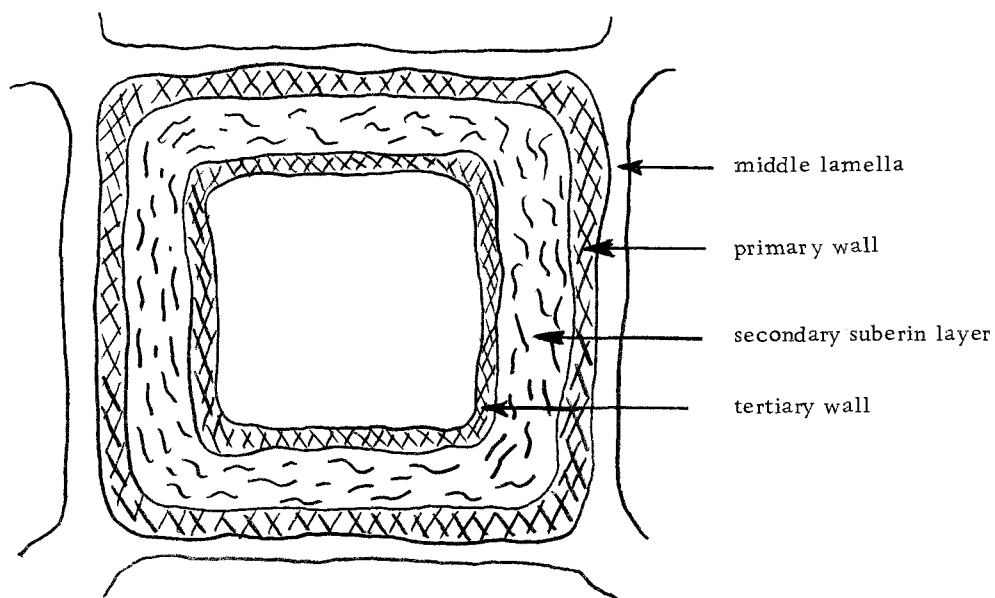
The ultrastructure of phellem cells has not been extensively studied. Crist (7) has examined the thick walled phellem cell found in longleaf pine (Pinus palustris mill.) while Sitte has studied the phellem cells found in bottle cork (Quercus suber) (37, 38, 39). Sitte has proposed the structure seen in Figure 5A for the cell wall in bottle cork (37, 38, 39). However, a tertiary cellulosic wall which may be lignified (as seen in Figure 5B) is sometimes present (10). The absence of this tertiary wall, as in bottle cork, allows for a more elastic wall. The wall of the cork cell according to Sitte (39) contains pits which are plugged with a substance "alien to suberin."

Krahmer and Wellons (23) have noted that thick-walled phellem cells can be observed in Douglas-fir. Their shape and size are

Figure 5. Proposed models of cork cell walls (37)



A. Two layer model



B. Three layer model

similar to the normal phellem cell but their wall is thickened considerably and stains red with phloroglucinol in dilute hydrochloric acid. This, they say, indicates that the walls may be lignified. They also noted that small pores or pits can be observed in these cells, while in normal thin-walled phellem cells neither a positive test with phloroglucinol nor pits can be observed. Grillos (13) and Chang (4) discussed the occurrence of thick-walled phellem cells in Douglas-fir and mentioned that they often appear in alternate bands with the thin-walled cells or sporadically distributed. When thick-walled cells are numerous, the material is referred to as "woody cork."

The chemical groups usually associated with cork are extractives, suberin and carbohydrates. Most chemical studies have centered around the two largest components (by weight), extractives and suberin. Hergert and Kurth (16) have reported that Douglas-fir cork contains from 30 to 40% extractives, while Kiefer and Kurth (21) have shown that the bast fibers of Douglas-fir bark contain only 13%. The major components of cork extractives were found to be dihydroquercetin, waxes, tannins and carbohydrate matter.

In extracting Douglas-fir bark, Fang (11) found esters of ferulic acid in the n-hexane fraction. Hergert (15), however, found ferulic acid after saponification of previously extracted white-fir (Abies concolor) cork. Hergert also mentioned in this paper that ferulic acid was found in Douglas-fir cork which has been extracted,

indicating that ferulic acid may be a component of the cork cell wall. However, the yield of ferulic acid was not mentioned. Because ferulic acid is similar to coniferyl alcohol, the proposed precursor to lignin, and because of the lack of extensive lignification in cork cells, ferulic acid might play an important part in the formation and structure of the cork cell wall.

Suberin was first described by Priestly (31) as an aggregate of variously modified forms (condensation or anhydrides) of certain organic acids which can be in combination with glycerine as true fats. The properties of suberin are described by Priestly (31) as 1) insoluble and impermeable to water, 2) considerably insoluble in organic solvents, 3) greatly resistant to concentrated H_2SO_4 , 4) readily oxidized by nitric acid or chromic acid, and 5) readily soluble in warm alkali.

Suberin has been studied only sparingly. Notable experiments on suberin from cork are those carried out by Zetzsche in the 1920's (44, 45, 46, 47) and by Jensen in the 1950's (18, 19). Neither were on Douglas-fir cork, but were on Quercus suber and Betula verrucosa, respectively. Both investigators extracted their purified cork in organic solvents, and then followed with a sodium sulfite extraction. After these steps the cork was saponified with alcoholic potassium hydroxide; yields were calculated and the solubles identified. Reported yields of suberin based on extractive free material are shown

in Table 1.

Some of the alkali solubles identified in Douglas-fir cork by Hergert and Kurth (16) were 11-hydroxy lauric acid, hydroxy palmitic acid, glycerol and a "phenolic acid" fraction.

The next step most investigators took was to place the residue from the saponification in acid. That material which was soluble was carbohydrate, and that which was insoluble was labelled "lignin." Besides yield data on carbohydrates some work has been done on the types of carbohydrates found in bark. A good bibliography on carbohydrate work done on bark can be found in Chen's thesis (5). Generally, most wood sugars have been found in barks also. In the inner bark a unique polysaccharide has been isolated which has been named callose; it is a β -D-(1 \rightarrow 3) - glucan (2). Callose has been found to plug the pits of sieve cells and epidermal cells (10).

Studies on the carbohydrates found in cork are rare. Hergert and Kurth (16) using paper chromatography found glucose and galactose; mannose and pentose sugars were not found to be present.

"True Wood Lignin" has not yet been substantiated in bark or cork. As previously mentioned the residue from the acid hydrolysis is considered to be lignin, but differences between wood lignin and bark or cork lignin have been shown to exist. Specifically the lignin isolated from bark has been shown to have a lower functional group value. The methoxyl content of lignin from bark has been shown to be

Table 1. Comparison of suberin contents found by various investigators on various species of cork

Investigator	Species	Yield % Extractive free basis
Zetzsche (44)	Quercus suber	35-44
	Robinia pseudacacius	6.3
	Fagus sylvatica	5.2
Jensen (18, 19)	Quercus suber	33.5
	Betula verrucosa (outer bark)	81.9
Holloway (17)	Ribes Americanum	30
	Ribes nigra	23
	Ribes grossularia	16
	Ribes davidii	31
Hergert & Kurth (16)	Douglas-fir	85.63*

*not a true determination of suberin content by the Zetzsche method but is actually the % of extractive free cork solubilized in .5N alcoholic potassium hydroxide after refluxing 9 hours.

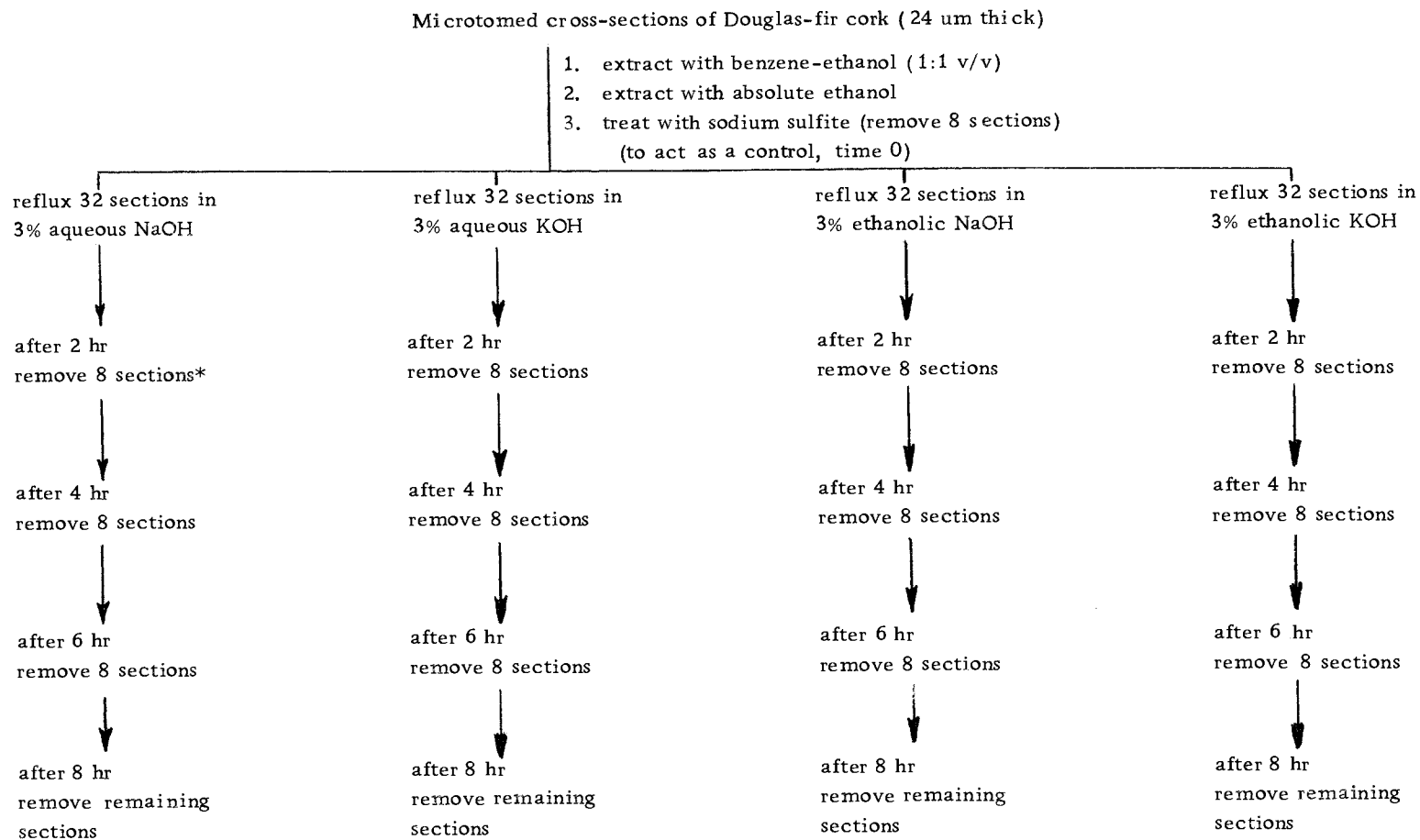
significantly lower than that of wood from the same species (15). Also Kiefer and Kurth (21) have compared different methods of isolating lignin from bark and have shown that these different methods yielded substantially different products with regard to functional group analysis. This gave rise to their conclusion that bark lignin was not a homogenous material (21). Although some important facts are known about bark and cork chemistry, the chemistry of bark, and specifically cork, is still in its infancy.

DESIGN OF EXPERIMENT

A search of the literature shows researchers using various conditions to saponify their cork (15, 16, 17, 18, 29, 44). This reaction is designed to strip away the suberin layer but it is very harsh and non-specific, and it can dissolve the entire cell if not properly controlled. It was therefore determined that before the major experiment could be undertaken, a smaller experiment to analyze the parameters of the saponification reaction on Douglas-fir cork had to be undertaken. An experiment to determine the effect of solvent, type of alkali, and time was designed and run as shown in Figure 6.

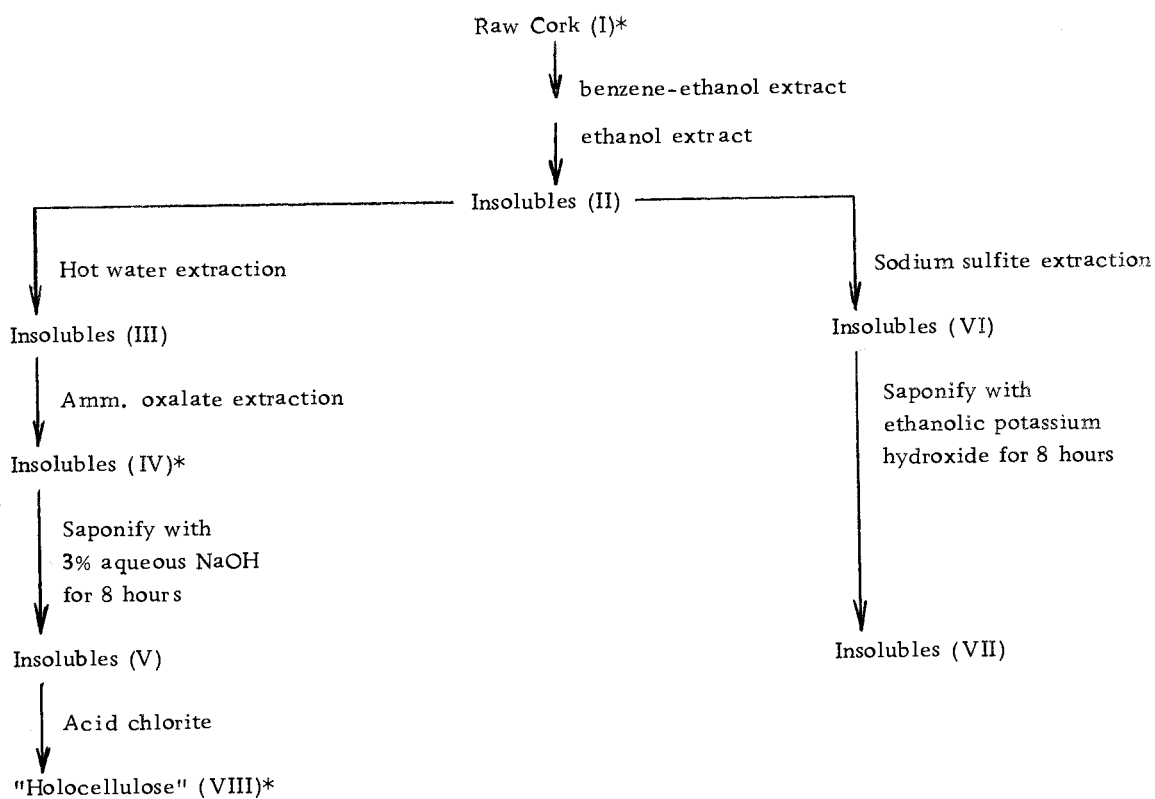
After obtaining the results from the above study the major experiment was undertaken. The basic approach to this experiment was to take 100% pure Douglas-fir cork and chemically treat it according to the flow sheet presented in Figure 7. At each step in the process, designated by a roman numeral, samples were removed so that various assays could be conducted to monitor changes in the cork. The assays conducted were specific gravity, ash, trace mineral (for Ca, Mg, Cu, Zn, Mn) and nitrogen by the Kjeldahl method. Besides these, paper chromatography was employed on the water soluble fraction of the benzene-ethanol extractives, the alkali saponifiables, and on acid hydrolyzates of samples from positions I, IV and the "holocellulose" fraction.

Figure 6. Procedure used to study the parameters involved in the saponification reaction



*After sections were removed they were washed and stored in distilled water. For an assay procedure, sections were stained with an IKI solution (34) and mounted on a glass slide. While observing the stained sections under a light microscope the sections were irrigated with 77% H_2SO_4 and the color changes in the section noted. (Suberin stains yellow to brown cellulose blue (34).)

Figure 7. Chemical treatments given to Douglas-fir cork



() - Each fraction at these points subjected to various assays to monitor changes in cork. Assays include specific gravity, trace mineral, ash, protein by Kjeldahl.

()*- Denotes positions where strong acid hydrolysis samples taken for carbohydrate analysis by paper chromatography.

Paralleling these chemical assays, microtomed sections of Douglas-fir cork were chemically treated in a similar manner as shown in the flow sheet of Figure 7. At each spot designated by a roman numeral, cork sections were removed and examined by various types of microscopy to monitor changes in the cork cell. The types of microscopy used were light, polarization, ultra-violet (U. V.), scanning electron (S. E. M.) and transmission electron (T. E. M.). In addition to studying cork sections, replicates of cork also were made and observed with T. E. M. Details on the chemical and anatomical techniques are given in the "Experimental" section at the end of this thesis.

RESULTS AND OBSERVATIONS

The results and observations section of this thesis is divided into 3 categories: 1) the saponification study, 2) chemistry and 3) anatomy. The chemistry and anatomy sections are further divided into subtopics, such as extraction, saponification, ultra-violet, transmission electron microscopy, etc. In this section, the specific information from each of these experiments or techniques is given and analysed. Later in the discussion section of this thesis the results are integrated to give a description of the phellem cell wall.

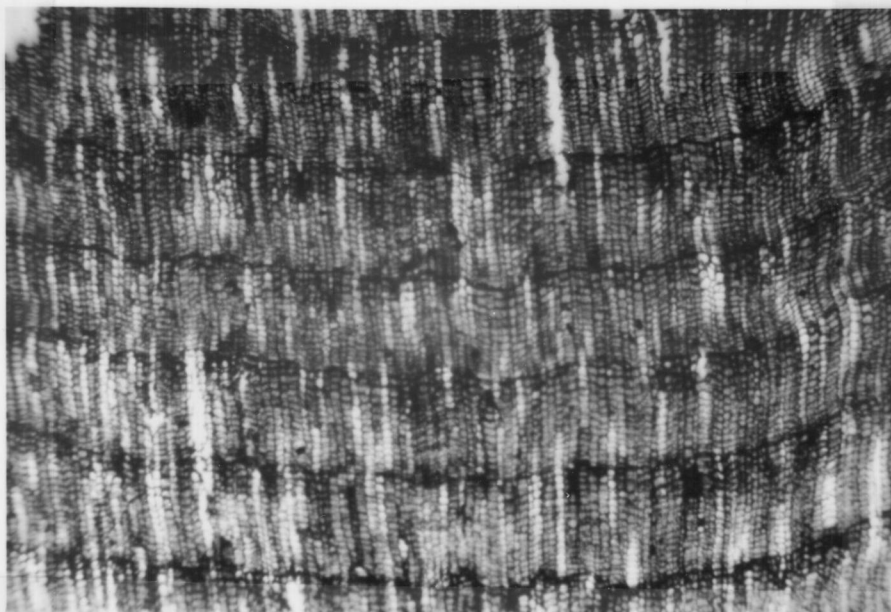
Effect of Time, Alkali and Solvent in the Saponification Reaction

This experiment was designed to determine how long the reaction should be run in a specific system of alkali and solvent. It also yielded information on the effects of alkali and solvent on the cork.

The results of this experiment are shown in Figures 8, 9, and 10. Figure 8A is an unsaponified stained control section.

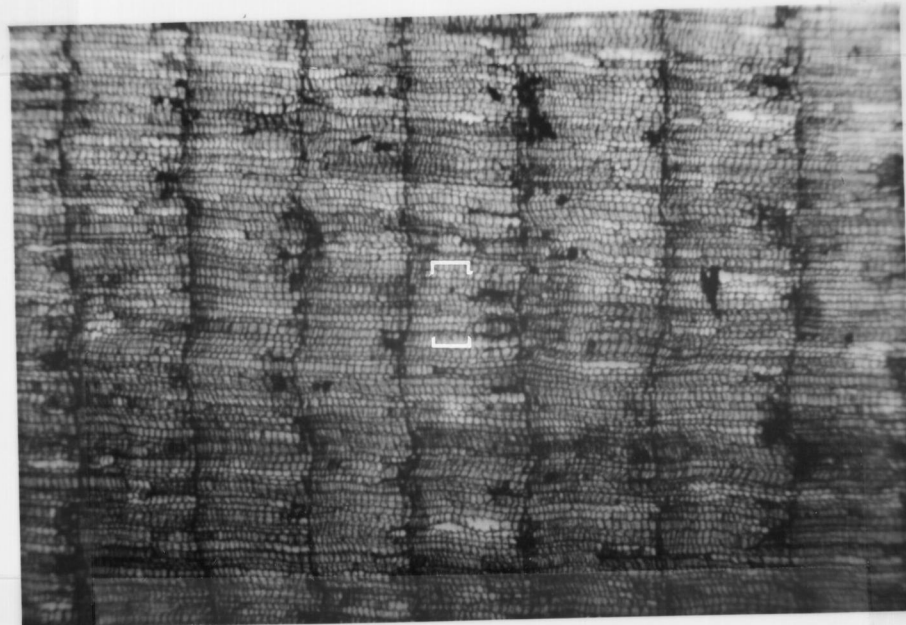
Figure 8B-8E are sections treated with aqueous sodium hydroxide for 2, 4, 6 and 8 hours, respectively. Areas stained with IKI and which appear yellow to brown are "suberin" (34, 35), while those appearing blue are cellulose (34, 35). These pictures show that after 2-4 hours of refluxing, the areas designated by the brackets are starting to stain blue, thus enough of the suberin secondary wall has been

Figure 8. Effect of time in the saponification reaction



Picture A. Organic solvent extracted cork stained with IKI

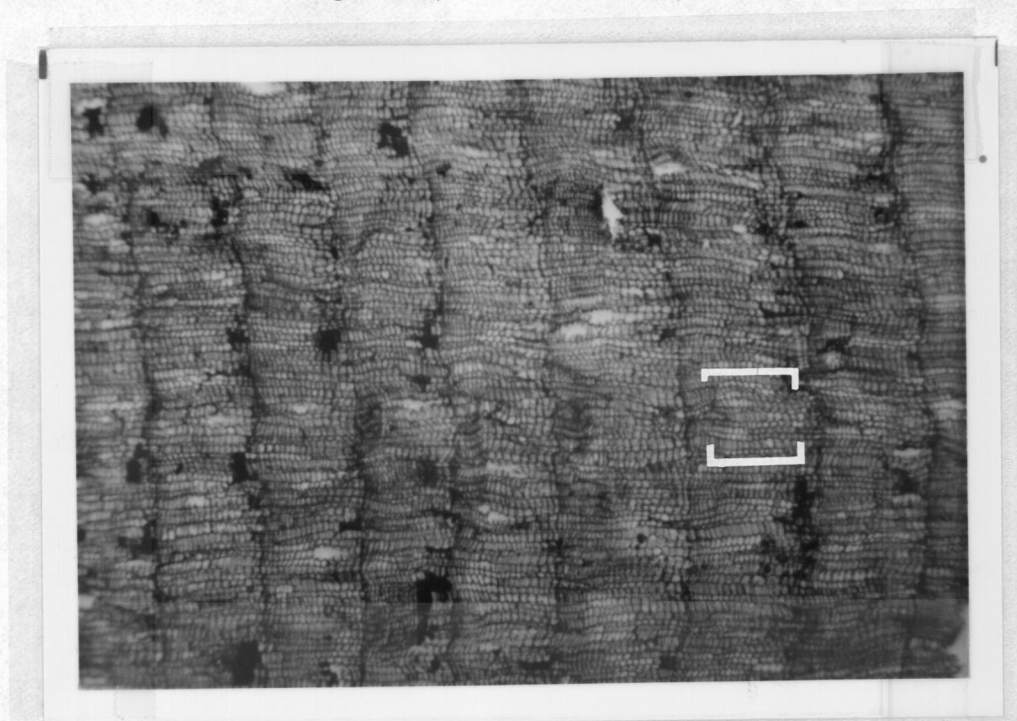
mag. 34X



Picture B - after 2 hours in alkali

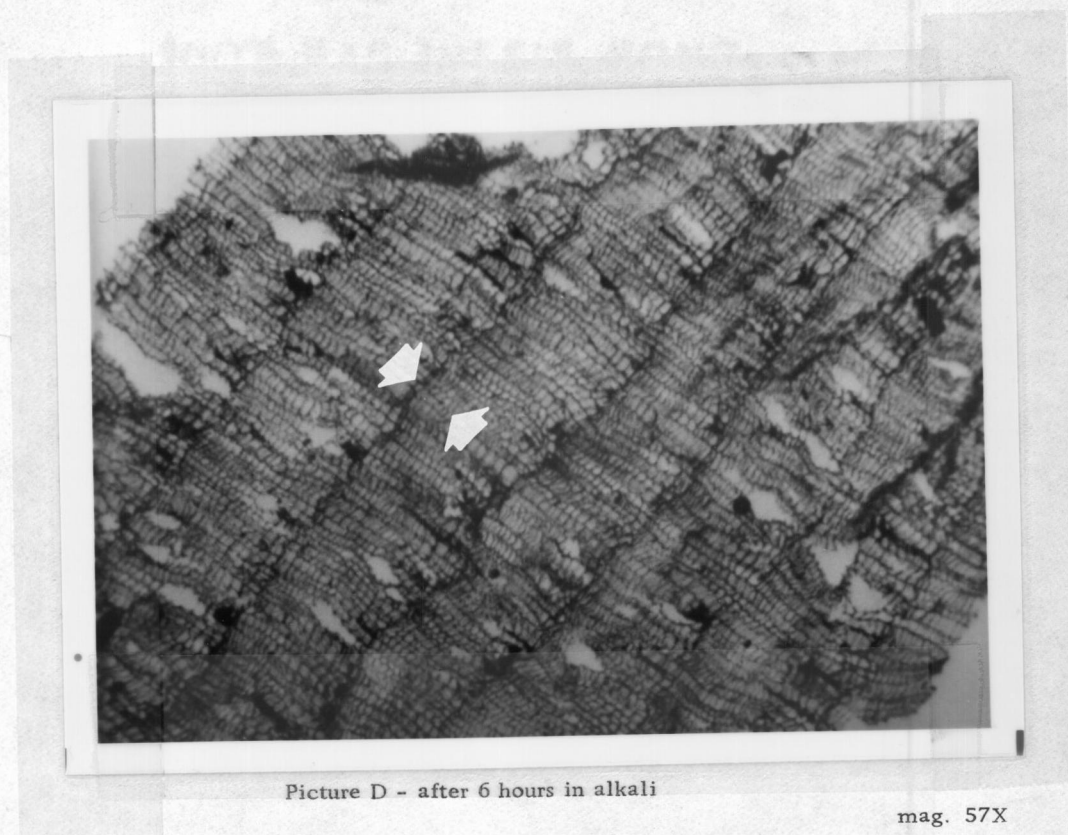
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Figure 8. (Continued)



Picture C - after 4 hours in alkali

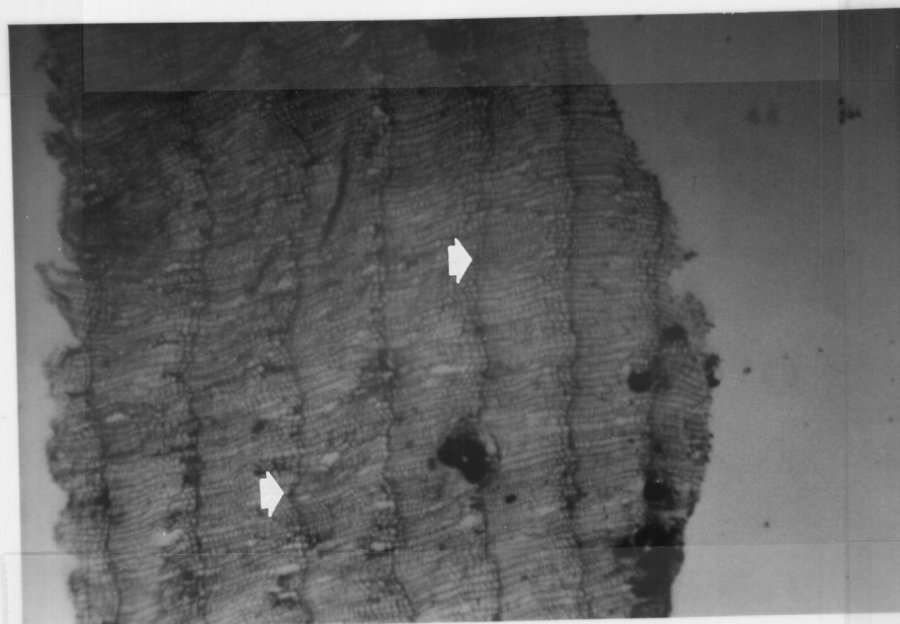
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Picture D - after 6 hours in alkali

mag. 57X

Figure 8. (Continued)



Picture E - after 8 hours in alkali

mag. 35X

removed to reveal the cellulosic primary wall.

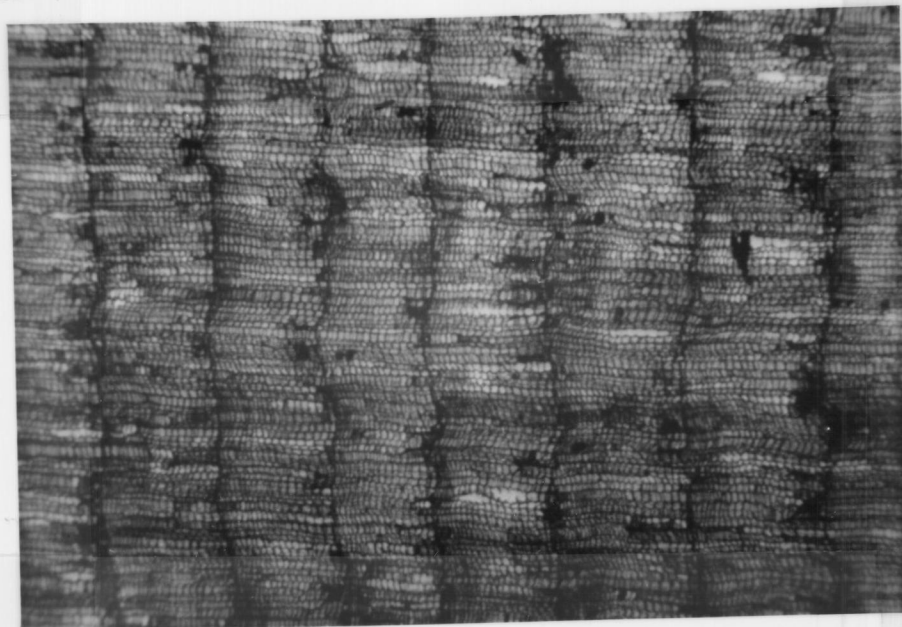
However, one should note that the area in a "growth increment" where the cork cells have been crushed still stains brown indicating that some suberin remains.

Prolonged refluxing (6-8 hours) has the effect of removing almost all suberin from all cork cells present in a growth increment. However, as before, there seems to be an area at the junction of the last crushed cell and the first uncrushed cell (designated by an arrow) which still stains brown.

There are possibly two reasons why this area still stains brown after 8 hours of refluxing: 1) there is an extremely thick layer of suberin present in this area and a longer reaction time is required to remove the suberin or 2) the material staining brown may or may not be suberin, but is very alkali resistant. Sections refluxed for more than 8 hours fell apart and therefore could not be observed. The second possibility seems most plausible because of the insignificant differences exhibited between the 6 hr and 8 hr treatments. Later results will show that 85% of the material solubilized in 8 hrs comes out in the first 2 hours. For these reasons 8 hours of refluxing in aqueous sodium hydroxide was considered to be of sufficient length to remove any suberin present in the cork.

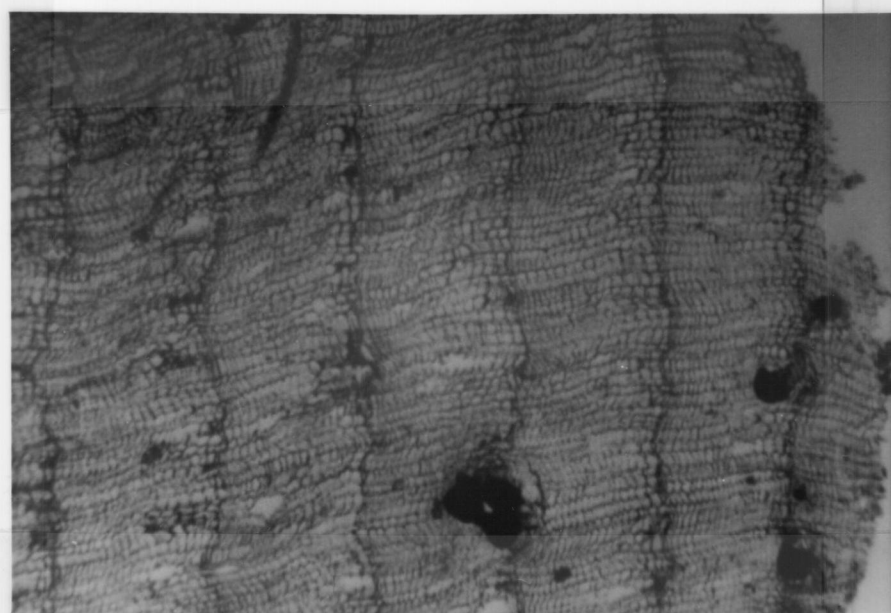
The second variable studied, the effect of alkali, is shown in Figure 9. Figure 9A and 9B are for aqueous sodium hydroxide at

Figure 9. Effect of alkali in the saponification reaction



Picture A - after 4 hours in aqueous sodium hydroxide

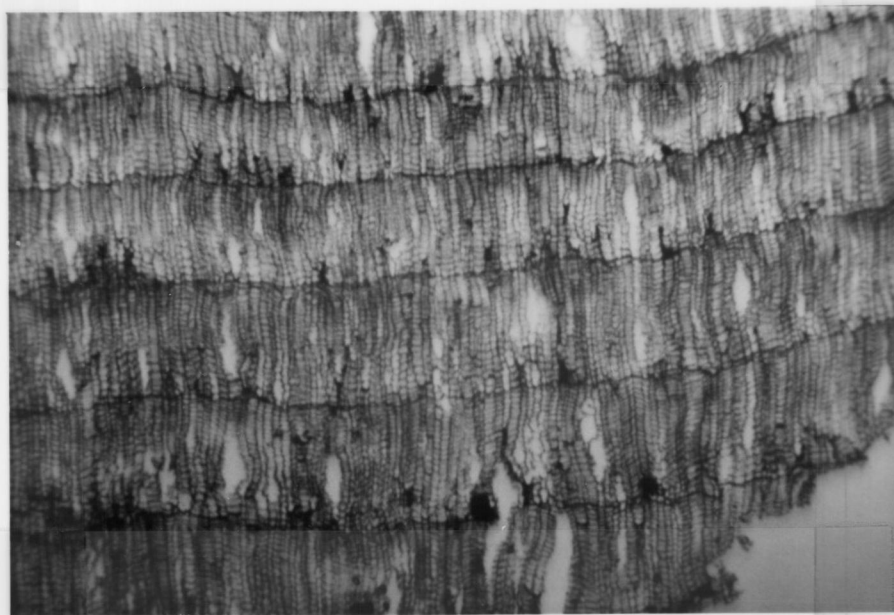
mag. 33X



Picture B - after 8 hours in aqueous sodium hydroxide

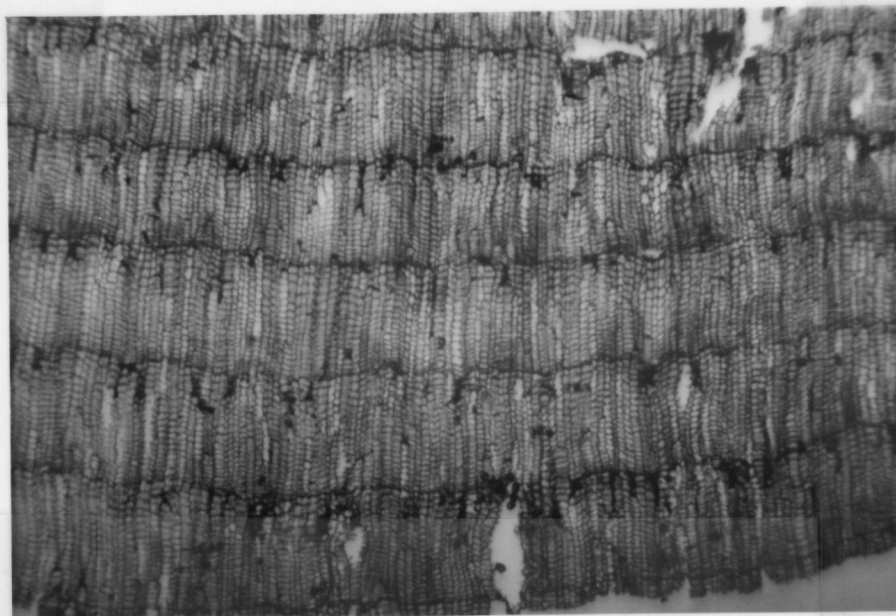
mag. 38X

Figure 9. (Continued)



Picture C - after 4 hours in aqueous potassium hydroxide

mag. 35X



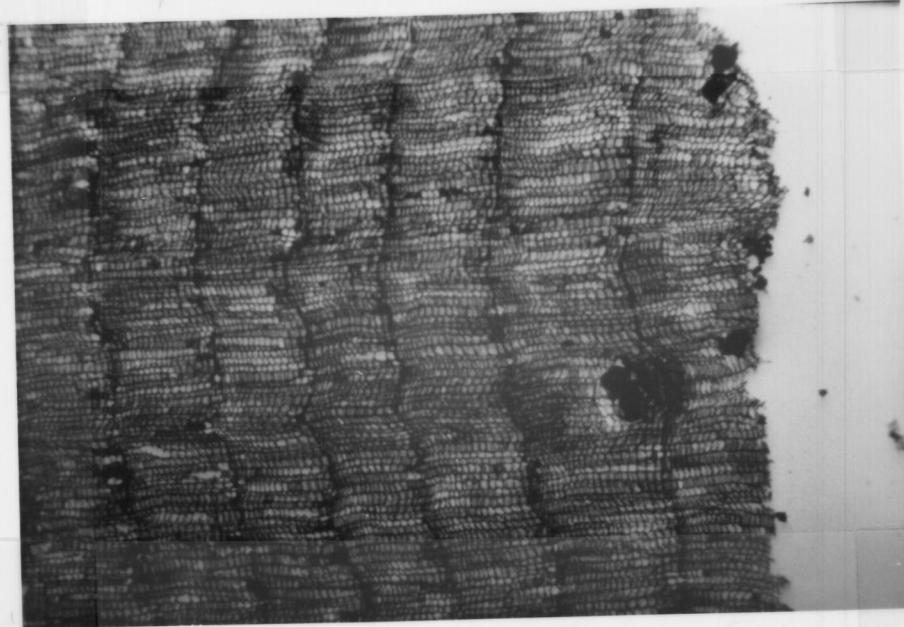
Picture D - after 8 hours in aqueous potassium hydroxide

mag. 33X

4 and 8 hours of refluxing, respectively. Figures 9C and 9D are for aqueous potassium hydroxide at 4 and 8 hours, respectively. In comparing the 4 hour treatments noticeable differences can be seen with respect to the amount of blue coloring or cellulose showing (A showing more blue than C). At 8 hours there is still a difference. From this it seems that the sodium hydroxide reacts faster than the potassium hydroxide. This is not unexpected since potassium hydroxide is considered to be a milder alkali than sodium hydroxide.

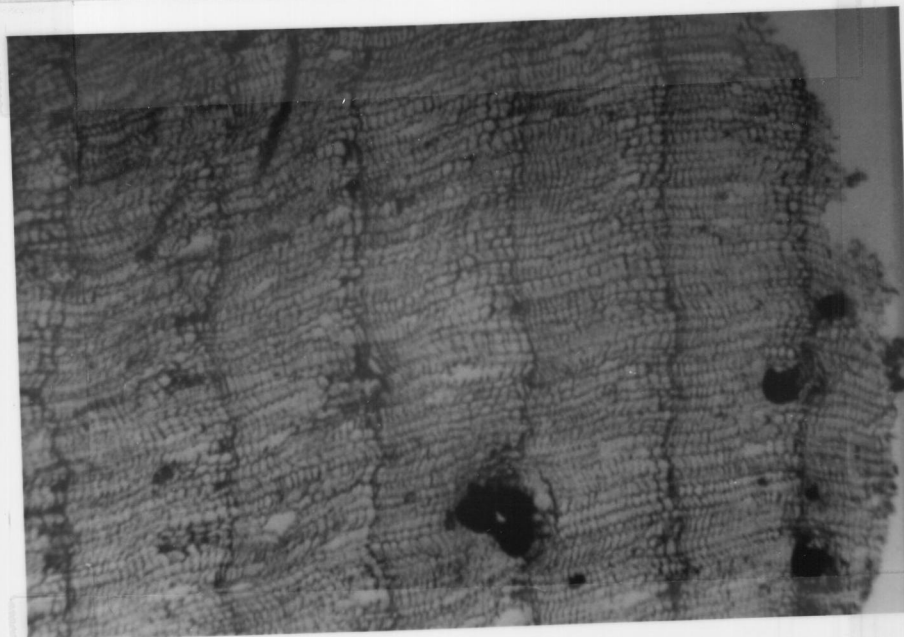
The last variable studied the effect of solvent (an aqueous solvent versus an ethanolic solvent), seems to have produced the greatest effect. Comparing Figures 10A and 10B to 10C and 10D (aqueous sodium hydroxide at 2 and 8 hours to ethanolic sodium hydroxide at 2 and 8 hours) two things can be noticed. First the sections in the ethanolic solvent appear to have been torn apart, especially after the 8 hours treatment. Second, very little of the cork from the ethanolic treatments stains blue, indicating poor removal of suberin. Later it will be shown that the IKI stain does not work with sections saponified in ethanolic solvents and the amount of suberin removed from these treatments cannot be monitored. However, the solvents do appear to be working in different areas of the cell wall, the aqueous solvent having its main effect on the removal of suberin while the ethanol treatment acts more like a macerating reaction. Because of these differences caused by the solvents it was

Figure 10. Effect of solvent in the saponification reaction



Picture A - after 2 hours in aqueous sodium hydroxide

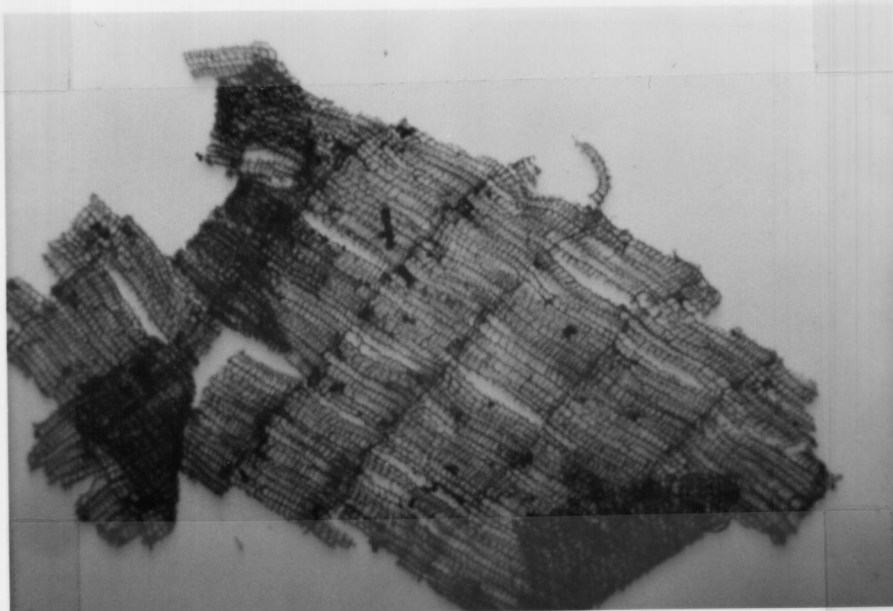
mag. 33X



Picture B - after 8 hours in aqueous sodium hydroxide

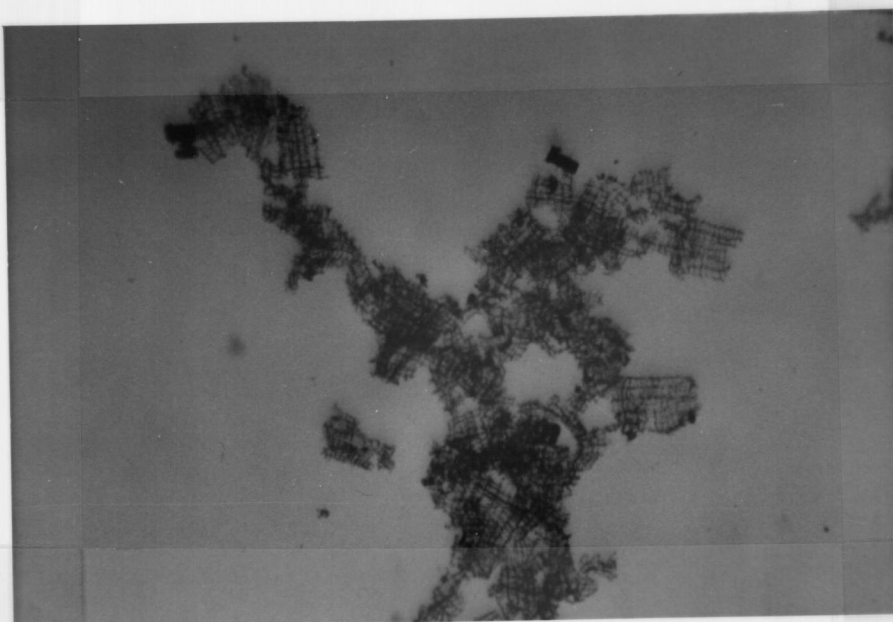
mag. 35X

Figure 10. (Continued)



Picture C - after 2 hours in ethanolic sodium hydroxide

mag. 35X



Picture D - after 8 hours in ethanolic sodium hydroxide

mag. 35X

determined that both should be used and that if differences in chemical composition could be detected, the differences might be related to differences in chemical composition between the suberin layer and the compound middle lamella. One alkali system selected was that of aqueous sodium hydroxide because of its efficiency in stripping the wall, and as shown, an 8 hour reaction time would be sufficient. The second system employed ethanol as the solvent and potassium hydroxide as the alkali. Potassium hydroxide was used because Zetzsche (44) and Meara (29) have employed such a system in similar studies. Eight hours was considered to be sufficient reaction time since only differences in chemical composition were being studied without trying to completely remove the suberin layer.

Chemistry

Yield Data for the Extractions and Chemical Treatments

Figure 11 shows the amount solubilized by various successive chemical treatments of a 100 gram o. d. batch of ground cork. The largest quantities of components of Douglas-fir cork are shown to be the benzene-ethanol extractives, which account for 47.75 grams/100 grams of cork, and the alkali solubles, which account for 26 grams/100 grams of cork. By weight these two fractions account for over 70% of the cork cell. Because they represent such a large percent

Figure 11. Chemical components present (by percent) in the Douglas-fir phellem cell.

Aqueous sodium hydroxide		Ethanolic potassium hydroxide	
Benzene-ethanol	47.75	Benzene-ethanol	47.75
Ethanol	3.45	Ethanol	3.45
Water	2.65		
Ammonium oxalate	15.17	Sodium sulfite	16.01
Saponification with 3% aqueous NaOH	26-27	Saponification with 3% ethanolic KOH	26
Unsaponified	3-4	Unsaponified	6-7

of the cork cell, further analysis of these two fractions was undertaken.

Analysis of the Water Soluble Fraction of the Benzene-Ethanol Extractives

It was found that 20% of the benzene-ethanol extractives were water soluble. Therefore 9.55 grams of the 47.75 grams removed/100 grams of cork was water soluble. Of this 9.55 grams, 5% or .48 gram was carbohydrate by the phenol method (43).

Paper chromatography of the water solubles revealed three spots. Two of the spots co-chromatographed with glucose and xylose, the third spot moved with the solvent front. Aniline hydrogen phthalate, a spray reagent for sugars, turned the one spot which co-chromatographed with glucose and authentic glucose brown. It turned the one spot which co-chromatographed with xylose and authentic xylose red. The spot running with the solvent front did not react. Ferric chloride spray reagent however indicated that this spot was phenolic. No di- or tri-saccharides were found in this extract.

Kurth (16) found for Douglas-fir cork an organic solvent extractives content of approximately 30-40% while my values approach 50%. This discrepancy could be caused by differences in collection, isolation and processing of the raw cork. For my study, only thick

periderms from old growth logs were sampled, and the cork was isolated by excising the periderms by hand. This allowed for a 100% pure cork fraction as a starting material. Kurth's starting material, because of his mechanical isolation process, was probably contaminated with materials such as bast fibers and parenchyma cells which would not have a high extractives content.

Also, Kurth's starting material was ground to a fine size at elevated temperatures. This might have the effect of lowering the extractives content through the fact that grinding at elevated temperatures could lead to fractures along the cell wall and separation of intact cork cells. Solvents would then have to move through the cell wall in order to get to the extractives. After dissolving the extractives, the extractives would then have to move through the cell wall and this may be a very slow process or may be impossible for some extractives. The inability of the extractives to move out of the cell could then lead to lower values for extractive contents. By grinding frozen material, brittle fractures across the cell wall were produced, thus eliminating the above problem by opening up the cell. Proof that the fractures were across the cell wall was obtained through the scanning and transmission electron micrographs.

One other reason for my high value of 50% is that this value includes some contaminating carbohydrate material. The possible origins of these carbohydrates is rather interesting. If we assume a

fracture across the cell wall and if the cork cell is as proposed in Figure 5A, then the carbohydrates must come from the primary wall-middle lamella region, which is possible. However, if the wall is comparable to that shown in Figure 5B then the carbohydrates may have originated from either the cellulosic tertiary wall and/or the compound-middle lamella region. Therefore in looking for a tertiary wall it is only relevant to look at unextracted cork sections.

Analysis of Alkali Saponifiabiles

From Figure 11 it can be seen that approximately 84% of the extracted cork cell wall is alkali soluble. For both the aqueous sodium hydroxide and the ethanolic potassium hydroxide this 26 grams/100 grams of raw cork was split into three fractions based on pH. Then each of these three fractions was split into two fractions based on solubility in acetone. Using the figure of 26 grams/100 grams of raw cork as 100%, Table 2 shows the various percentages contributed by these six fractions (3x2) (i. e., that material which was precipitated out of the aqueous sodium hydroxide treatment at pH 6.5 and was acetone soluble accounted for 2.9% of the total 26 grams).

This table shows that there were considerable differences between an aqueous sodium hydroxide and the ethanolic potassium hydroxide saponification products. This is in agreement with what was found using sections of cork in the study on the saponification

Table 2. Analysis of alkali saponificables according to pH and acetone solubility.

Aqueous sodium hydroxide	Acetone solubles	Acetone insolubles	Total %
6.5	4.29	9.67	13.06
4.0	21.07	3.15	24.22
<4.0	61.78	trace	61.78
Total %	87.14	12.82	99.96
Ethanollic potassium hydroxide			
6.5	trace	22.56	22.56
4.0	43.17	3.35	46.52
<4.0	30.38	trace	30.38
Total %	73.55	25.91	99.46

variables. Thus the results of Table 2 are not unexpected.

It was hoped that paper chromatography could help indicate what chemicals were present in each of these fractions and that from this some conclusions could be made regarding lignification or cementing substances present in the cork cell wall. The results of chromatographing these fractions were inconclusive due to extensive streaking of the unknowns with some solvent systems. Much of the acetone soluble fractions are phenolic. "Ferulic acid like materials" were present throughout the acetone soluble fractions, and other phenolics which gave a yellow to green fluorescence under U. V. were also present in the acetone soluble fractions.

Protein and Trace Mineral

The protein and trace mineral analyses were done on cork residues from the chemical treatments. This part of the study was designed to monitor the loss of protein by noting the losses in nitrogen content.

In analysing the disappearance of protein it seems that the saponification reactions and the sodium sulfite reaction are most efficient in removing protein. This agrees with findings of Zetzsche (43). In absolute amounts, protein is shown to make up approximately 1% of the dry cork cell's weight.

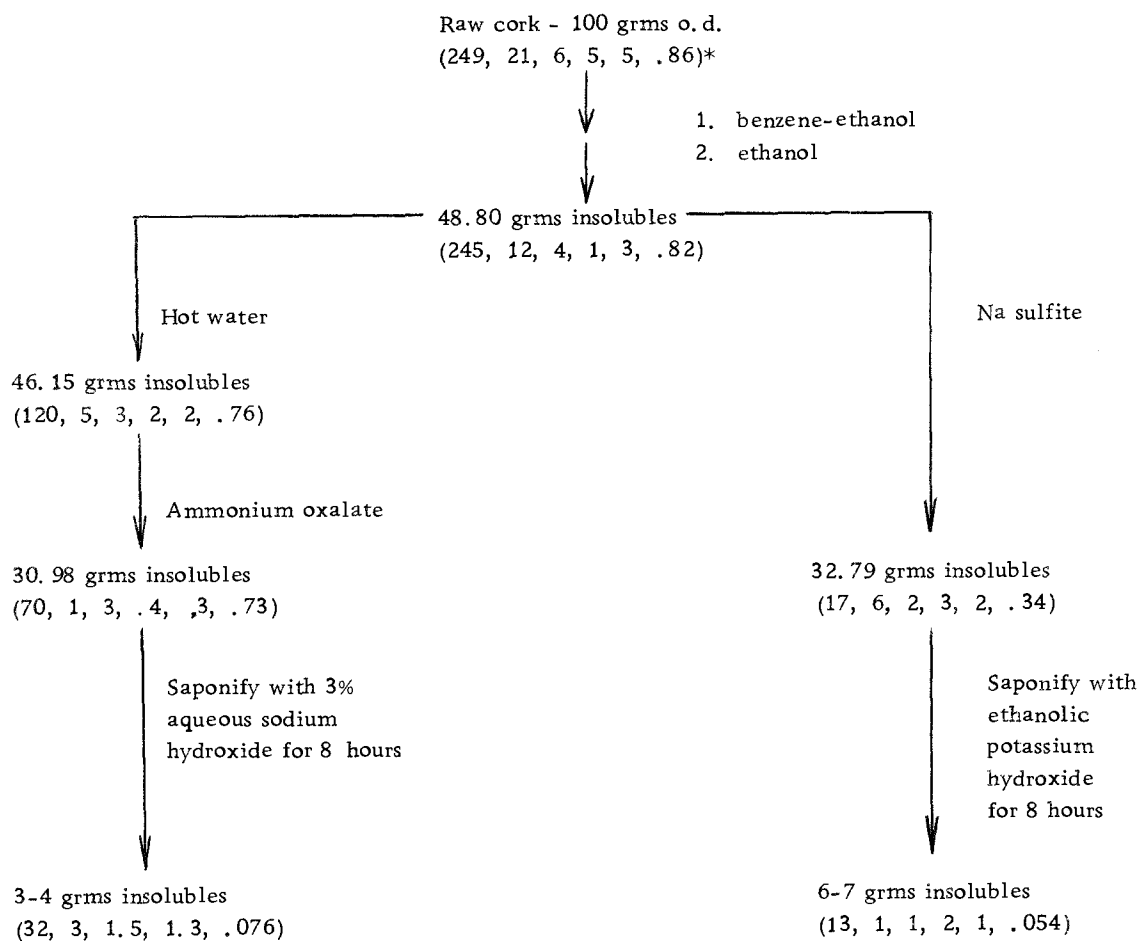
In analysing the disappearance of the trace minerals, only one

mineral, calcium, is present in large enough quantities to be monitored. The disappearance of calcium was an indication of a loss in pectins since pectins are chelated to calcium and magnesium (10). The results of these analyses are presented in Figure 12. It seems that organic solvent extraction has no noticeable effect on removing minerals. Hot water extraction however removed approximately 50% of the calcium present. This is expected because the hot water extraction removes pectins (1).

Ammonium oxalate treatment is a slightly harsher method of removing pectin as can be seen in Figure 12, a further loss of calcium is also evident. However, both of these treatments together are less efficient in removing calcium than the sodium sulfite treatment alone. The sodium sulfite treatment dropped the calcium levels in the wall from 245 μg to 17 μg .

In some cases the trace mineral content seems to have risen (i. e. magnesium from 1 to 3 after saponification with aqueous sodium hydroxide). Two possible reasons for this rise are 1) the low values of minerals present coupled with only 2 replicates makes these fluctuations within normal statistical variations and 2) the sodium hydroxide added to treat the cork contains slight mineral impurities and even though the treated material was dialysed some of these impurities might remain.

Figure 12. Results of protein and trace mineral analysis



*(Ca, Mg, Cu, Zn, Mn, Protein)

Trace minerals in ug

Protein in grams

Values average of two runs

Acid Hydrolyzates

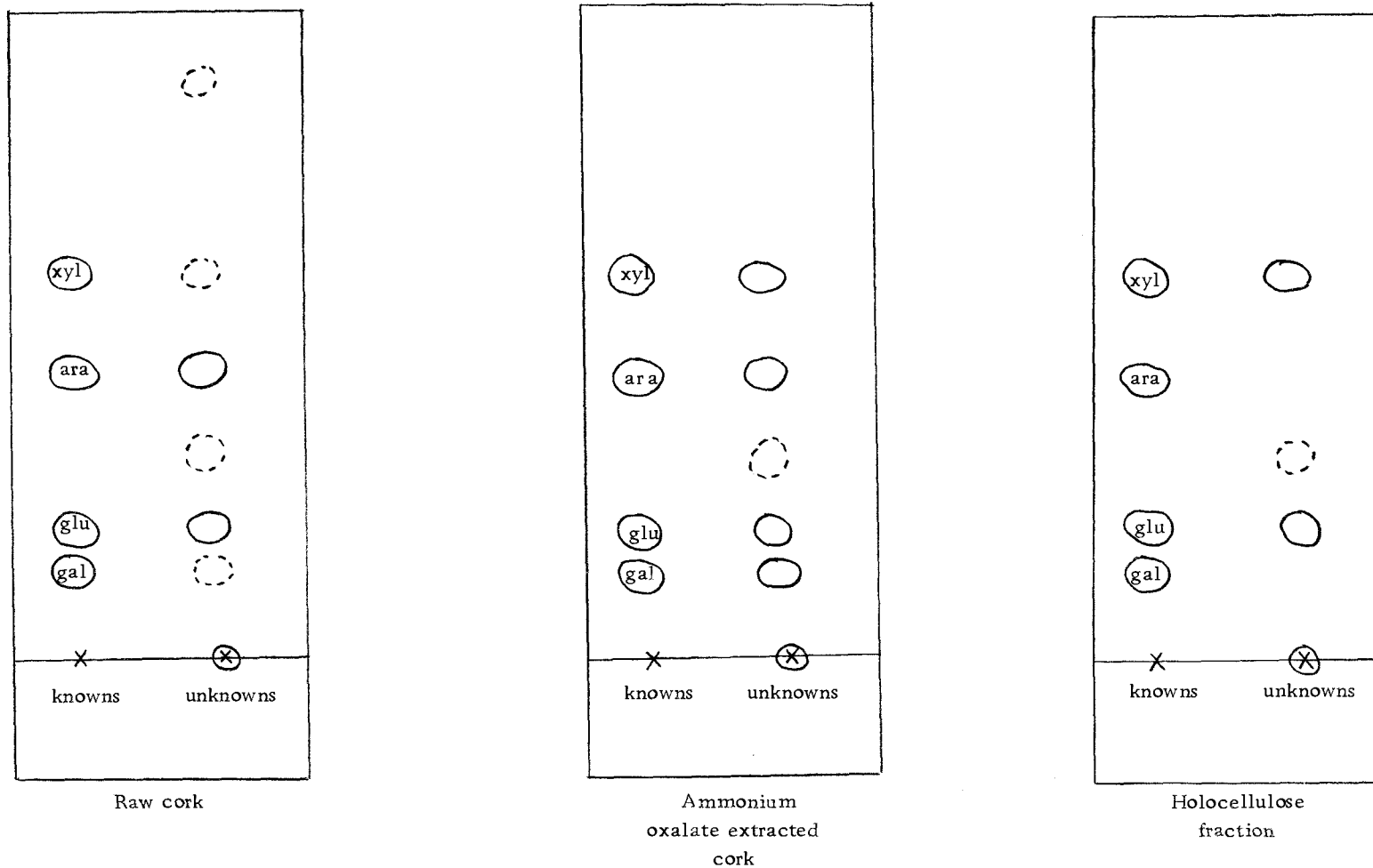
Acid hydrolysis of the various cork fractions was intended to produce information on the types of sugars present in cork, and to indicate if any of these sugars are lost because of the various chemical treatments the cork received. From the Experimental section (page 111) it can be seen that the starting weights of the various fractions were adjusted so that the concentrations of carbohydrates in the hydrolyzates would be approximately equal. This was done to allow comparisons to be made between the starting materials.

The results of chromatographing the hydrolyzates from the raw cork, ammonium oxalate extracted cork and the holocellulose fraction are shown in Figure 13. These data show that Douglas-fir cork possesses a full compliment of sugars. Also, all sugars were still present after the ammonium oxalate extraction, but galactose and arabinose are missing, or in such small quantities as to be non-identifiable, in the holocellulose fraction. These results parallel those of the trace mineral analysis in Figure 12.

Specific Gravity by Liquid Immersion

The method used in this part of the study detected density changes in the cork cell wall. Density changes were the result of removing certain cell wall components. These data can give valuable

Figure 13. Paper chromatographs of the acid hydrolyzates for detection of sugars. Solvent ethylacetate pyridine-water (8:2:1 v/v/v) spray reagent analine hydrogen phthalate (0 indicates major component, (○) indicates minor component)



information on the layering of the cell wall and on possible packing materials present in the cell wall.

The bulk density of cork is very low (42) but as shown in Table 3, wall density is high (1.84-1.89) especially when compared to xylem elements (1.4-1.5) (22).

After benzene-ethanol extraction, wall density is greatly decreased, showing that extractives have been acting as bulking agents in the wall. Extraction of these compounds has perhaps created micropores which could not be filled with the immersing liquid resulting in a lower wall density. With hot water extraction and ammonium oxalate extraction the wall density values increased. Possibly these extractions are causing enlargement of the micropores and the solvents subsequently fill these holes.

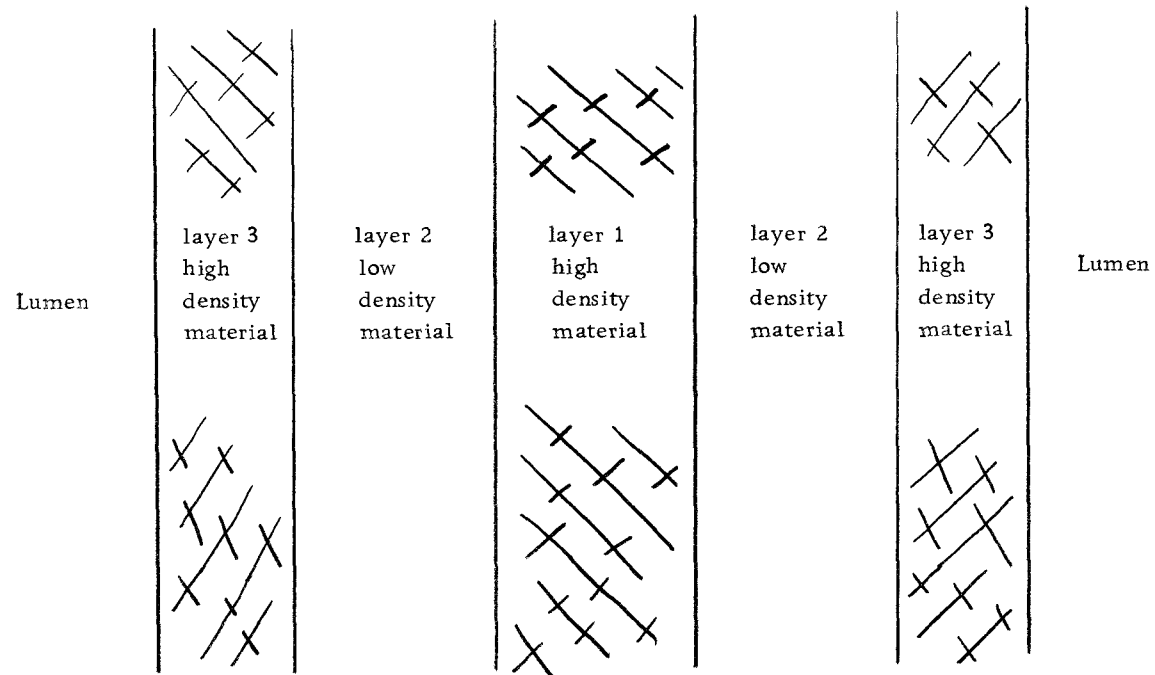
Another explanation of the above decrease and then increase in specific gravity with chemical treatment is possible. If the cork cell wall exists as alternating layers of high density and low density material (as seen in Figure 14), the stripping away of the dense material (layer 3) would result in an initial decrease in specific gravity. With further chemical treatment and the loss of layer 2, the specific gravity would increase. This possibility was checked by determining the specific gravity of the extract. The specific gravity of the benzene-ethanol extract was found to be 1.5-1.6 (see Table 3). These values are far too low to completely explain the above theory. However,

Table 3. Specific gravity of cork cell wall by the liquid immersion technique using four different liquids

Sample	Water	Ethanol	Acetone	Benzene
Raw cork	1.89*	1.94	1.70	1.46
Benzene-ethanol extracted	1.36	1.52	1.40	1.25
Benzene-ethanol ethanol extracted	1.48	1.45	1.42	1.33
Ammonium oxalate extracted	1.69	1.65	1.76	1.38
Benzene-ethanol extractives	1.51	1.61	1.47	1.44

*values are average of two runs

Figure 14. Diagram representing the double wall between two phellem cells



these values are higher than the specific gravity values found for the solvent extracted cell wall (1.3-1.5). Therefore, it seems that both of the above theories are valid to some extent and that the monitored drop in cell wall specific gravity and subsequent rise is the product of these two effects.

Also, it is interesting to note which solvent gives the highest density values (denoted by a circle around the value). High density values are indicative of the fact that solvent and wall material are mutually satisfying or compatible. After organic solvent, hot water and ammonium oxalate extraction, acetone gives the highest wall density value. Thus one would expect the cell wall to be more non-polar. This agrees with earlier moisture sorption work (27).

Because of a lack of material, samples representing treatments V, VI and VII could not be run. It would have been interesting to see what values these treatments would have given. Most likely, as further chemical treatments are given, the cork cell wall density will decrease to that of xylem elements and shift to water as the solvent giving the highest density values. This is due to the fact that the cell wall will become more cellulosic in character, with further chemical treatment.

Anatomy

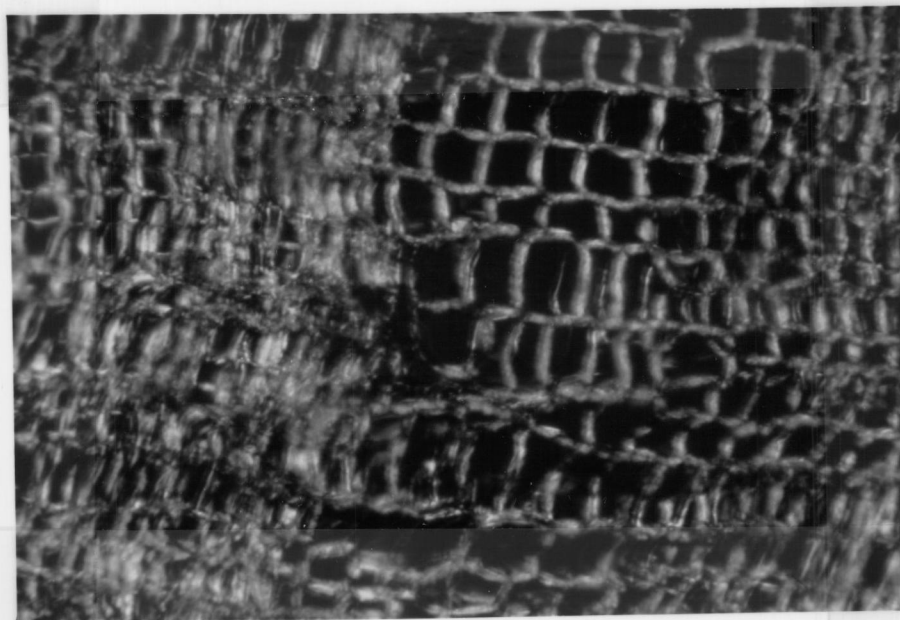
Polarization

The use of the polarizing microscope in conjunction with chemical treatments can give valuable information on the orientation of certain molecules in the cell wall. For the cell wall to be birefringent (appear bright in the figures) some molecules in the wall must be highly organized, and the longitudinal axis of these molecules must be parallel to the plane of the section. Those areas of the wall which appear dark must have the molecules oriented perpendicular to the plane of the section. The dark cross which appears on the macerated cells in Figure 16 is caused by the orientation of the polarizer and analyser and does not alter the interpretation of results. The results of the polarization study are shown in Figures 15 and 16.

Figures 15A-15D are cross sections of cork subjected to various chemical treatments. Figure 15A is of unextracted cork while 15B is of cork which had been extracted with benzene-ethanol and then ethanol. Both pictures show that the cork cell is birefringent. There is no discernible differences between Figure 15A and 15B.

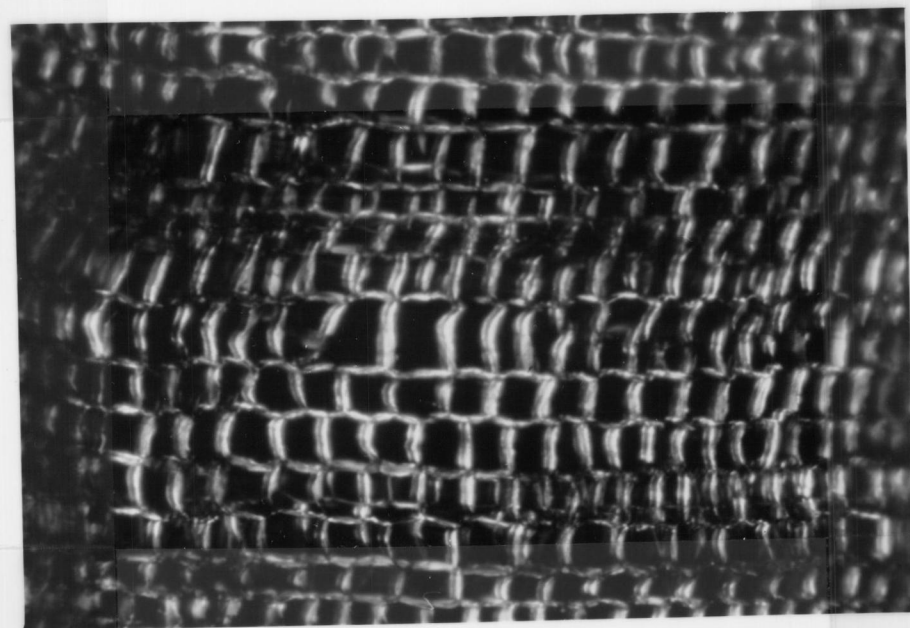
Figures 15C and 15D are of cork saponified with aqueous sodium hydroxide and sulfite treated cork respectively. What should be noticed in these photos is the loss of birefringent material. Until these treatments, there was no noticeable loss in birefringence.

Figure 15. Polarization pictures of Douglas-fir cork having various chemical treatments



Picture A - unextracted cork

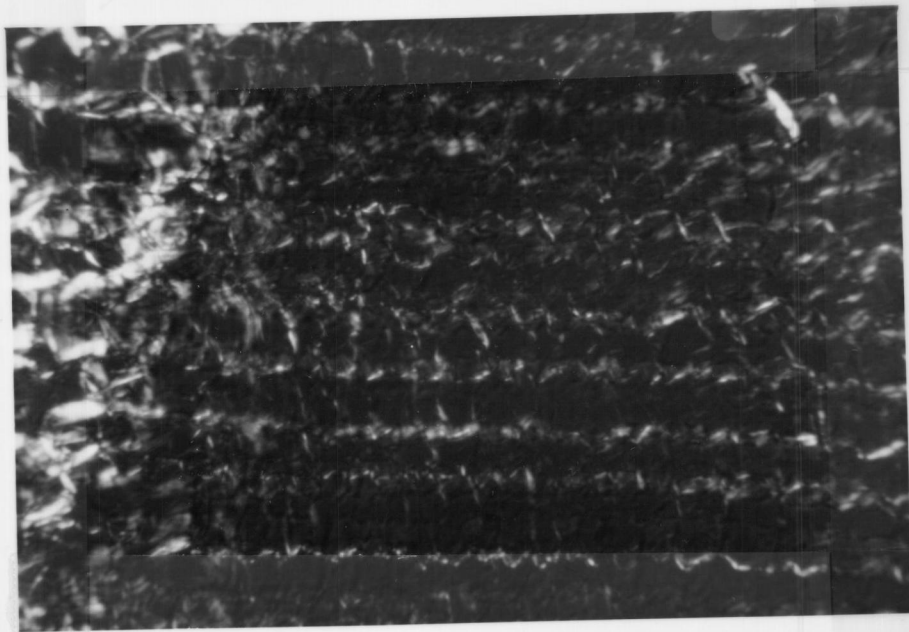
mag. 176X



Picture B - benzene-ethanol/ethanol extracted cork

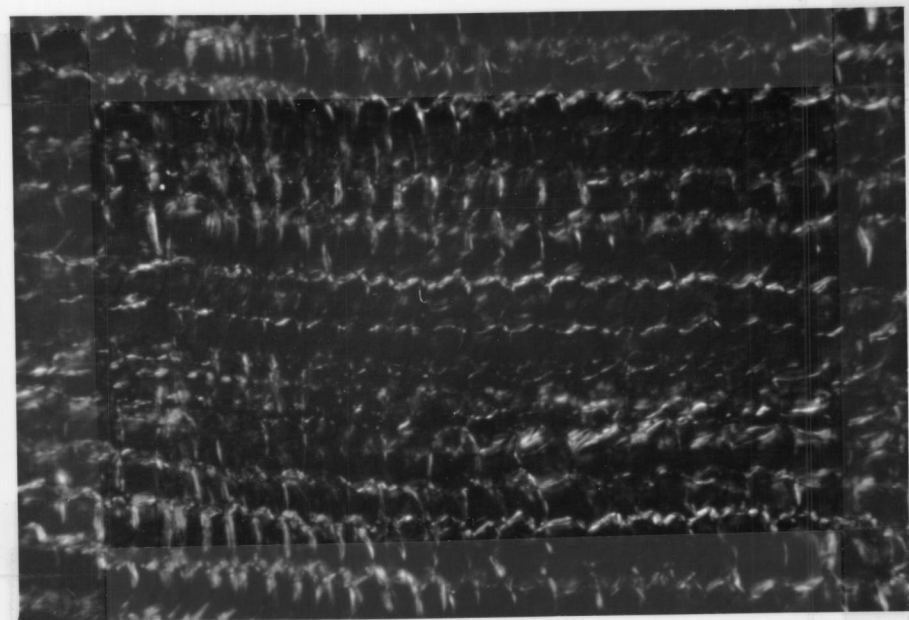
mag. 176X

Figure 15. (Continued)



Picture C - saponified 8 hours in aqueous sodium hydroxide

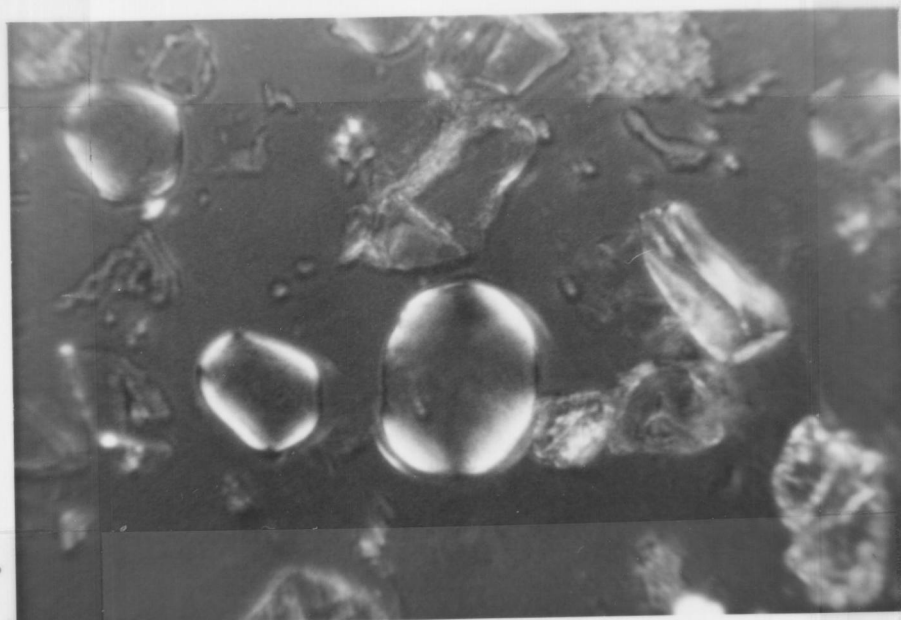
mag. 176X



Picture D - sulfite treated cork

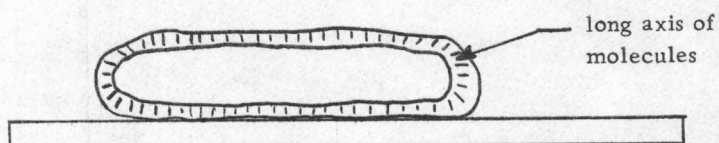
mag. 176X

Figure 16. Polarization picture of macerated cork and diagrams representing the cell in 16A.

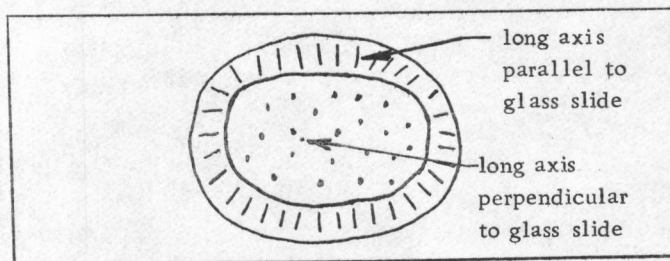


Picture A - Macerated cork

mag. 379X



Picture B. Side view as seen on the glass slide



Picture C. Top view as seen in the above photo

Thus the material removed by these treatments must be responsible for birefringence.

Figure 16A is of macerated cork cells under polarized light. Diagrams 16B and 16C show the orientation molecules causing the birefringence must have in order to produce the pattern of birefringence shown in 16A. Thus, the only possible orientation the molecules could have to produce the birefringence shown in 16A, is to be oriented perpendicular to the cell surface.

Ultra-Violet

The use of invisible ultra-violet light in the microscope has little value unless the material being studied fluoresces in the visible wavelengths. For an organic molecule to autofluoresce (not require a dye or stain to fluoresce) the molecule must possess valance or π electrons which are available for excitation. Also "among organic molecules fluorescence is to be expected in those species with large rigid multicyclic structures" (9). In some cases molecules which do not fluoresce can be observed by being stained or paired with a molecular species which does fluoresce. With callose an unusual phenomona occurs in that separately neither callose nor the stain analine blue fluoresce, but when callose is stained with analine blue a fluorescence in the visible region is created.

The results of the ultra-violet microscopy (U. V.) are shown

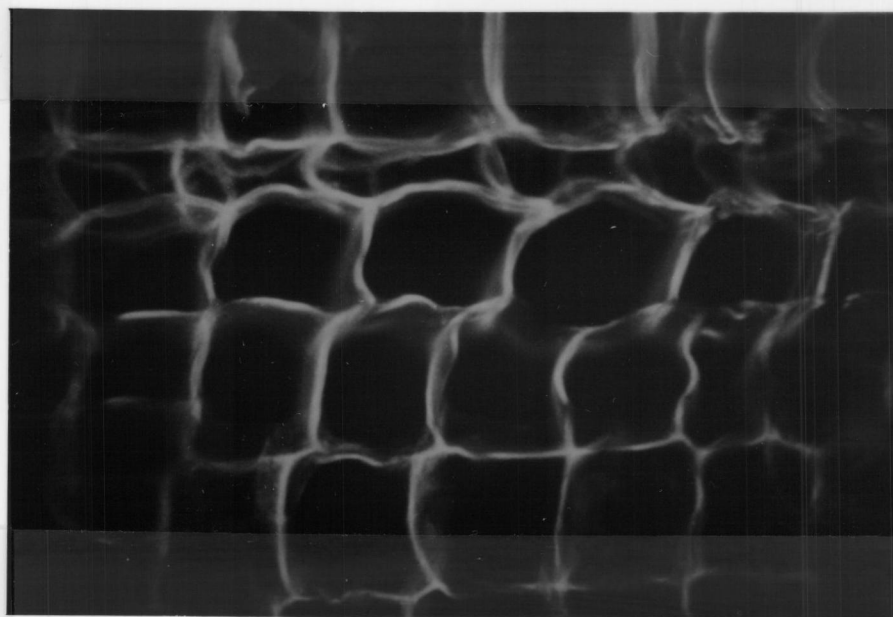
in Figures 17A-17E. These U. V. micrographs parallel those of Figure 15 in that the organic solvent, water and ammonium oxalate treated cork sections are not discernibly different than the untreated cork sections. However, after treatment with sodium sulfite or saponification there are noticeable changes. The U. V. micrographs show changes in fluorescence thickness and color.

Figure 17A and 17B are of untreated and benzene-ethanol, ethanol extracted sections which are not stained. Note that the sections exhibit autofluorescence and that the visible color being emitted is a yellow-green.

Figure 17C is of an unstained section having been treated with aqueous sodium hydroxide for 8 hours. In comparing 17C to 17A and 17B one can see a decrease in wall thickness and the color now appearing is a yellow-orange. Also in 17C one should note the large band of intense color coming from the area designated L. This is located at the junction of the last crushed cell in a growth ring and the first uncrushed cell in an adjacent growth ring. If one were to observe a comparable area from 17A or 17B, one would see a band of intense color but the actual color seen would be yellow-green not yellow-orange.

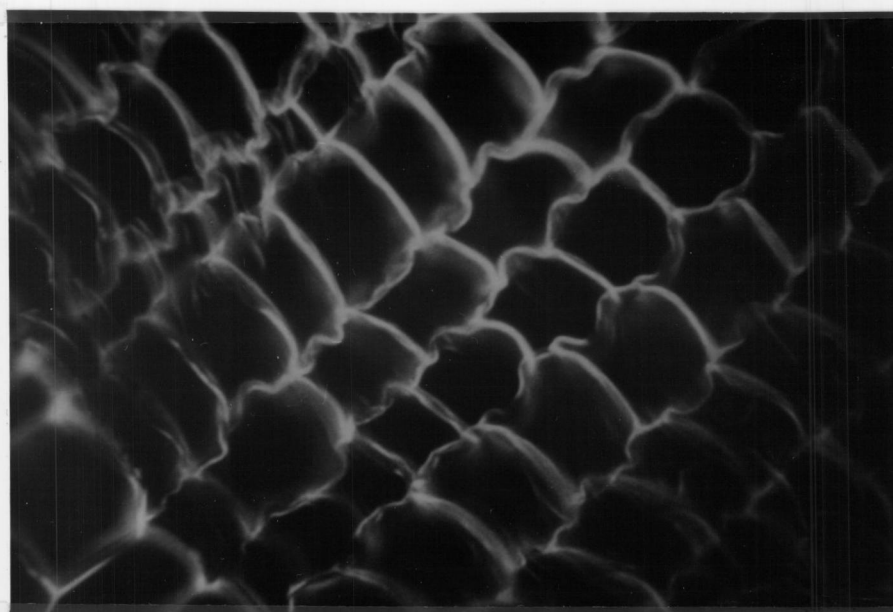
Figures 17C and 17D are of identical sections except 17D is stained with analine blue. The most important thing to observe in 17D is the appearance of the yellow-green dots. The appearance of

Figure 17. Pictures of Douglas-fir cork having various chemical treatments,
taken with an ultra-violet light source



Picture A - Unextracted, unstained cork section

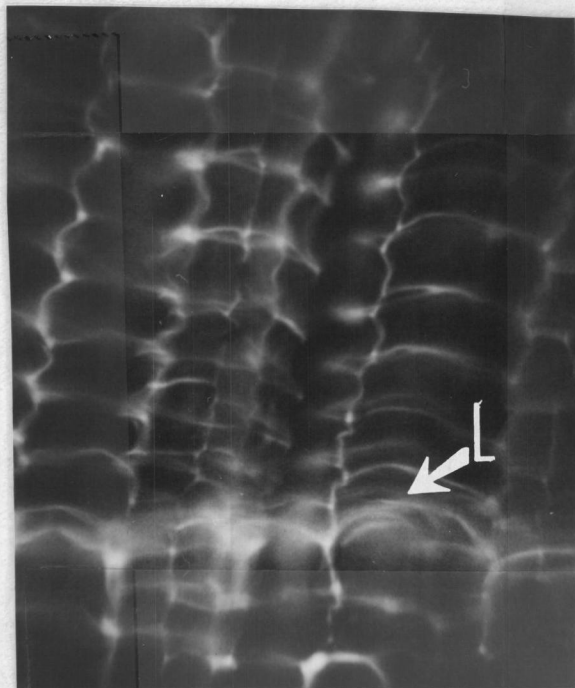
mag. 463X



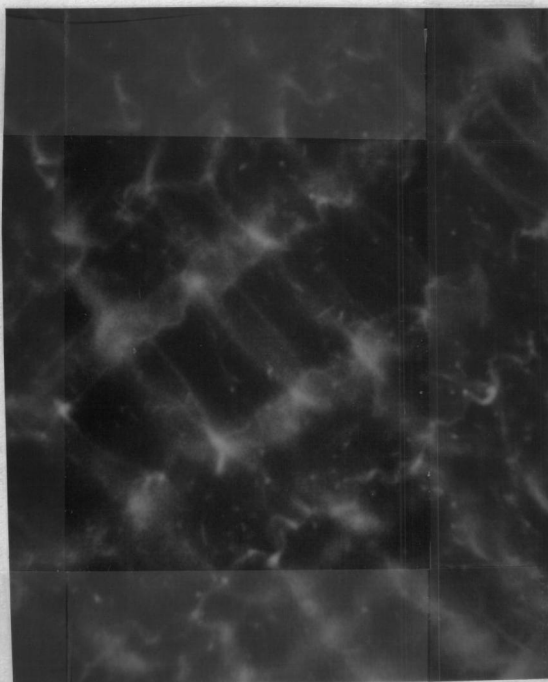
Picture B - Extracted, unstained cork section

mag. 472X

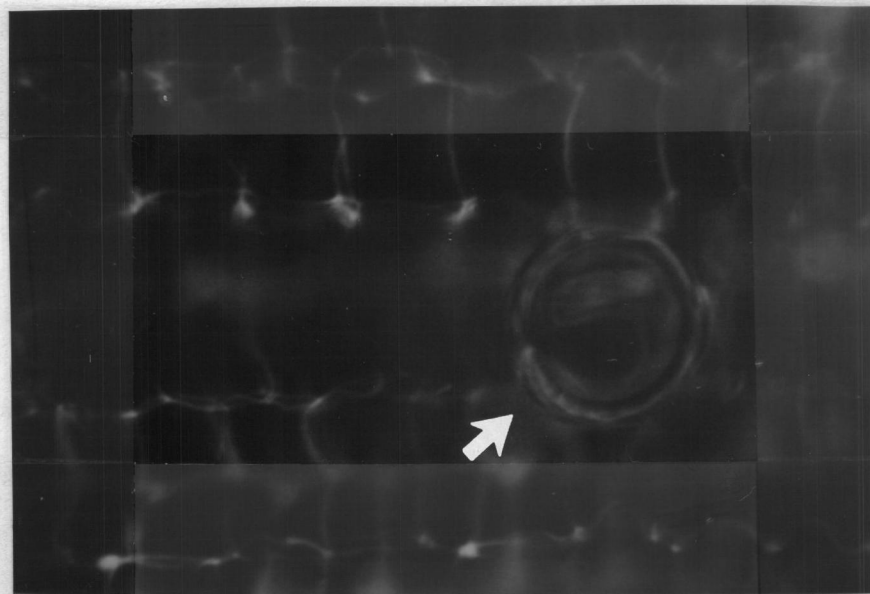
Figure 17. (Continued)



Picture C - saponified 8 hours with aqueous sodium hydroxide - unstained mag. 450X



Picture D - saponified 8 hours with aqueous sodium hydroxide - stained with analine blue (8) showing a callose reaction (green dots) mag. 450X



Picture E - unstained section with phenolic sheath surrounding bast fiber

mag. 460X

these dots indicate that they are callose (8, 10). These dots appear only after the saponification reactions. This indicates that 1) the callose is covered and is only observable after the saponification reaction has stripped away the covering material, or 2) the callose is masked and only after removing an autofluorescing material, which gives off a comparable visible color, is the callose made visible.

Figure 17E is a picture of a bast fiber surrounded by cork. The autofluorescing material which surrounds the bast fiber (designated by the arrows) is the phenolic sheath referred to by Ross (32). This picture is also evidence for the fact that cellulose does not fluoresce while phenolic material does.

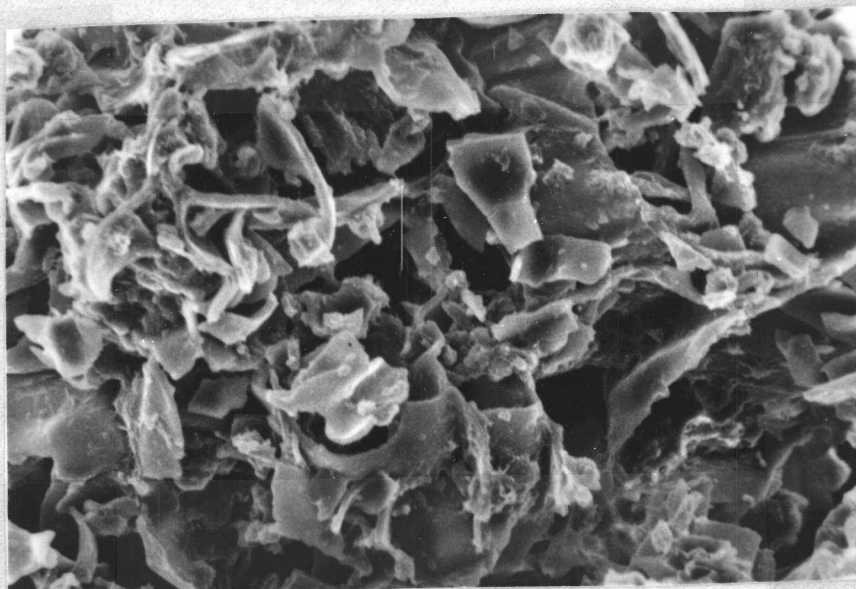
Scanning Electron Microscopy

The scanning electron microscope is a tool which can be used to study the surfaces of material at magnifications from 50X to 30,000X with a resolution of approximately 200 Å.

The results of the scanning electron microscopy work are shown in Figures 18 and 19. Figures 18A-18D are of the ground cork used in the chemical analysis and represent some of the chemical treatments. Figures 19A-19G are of cork sections which received chemical treatments comparable to the ground cork.

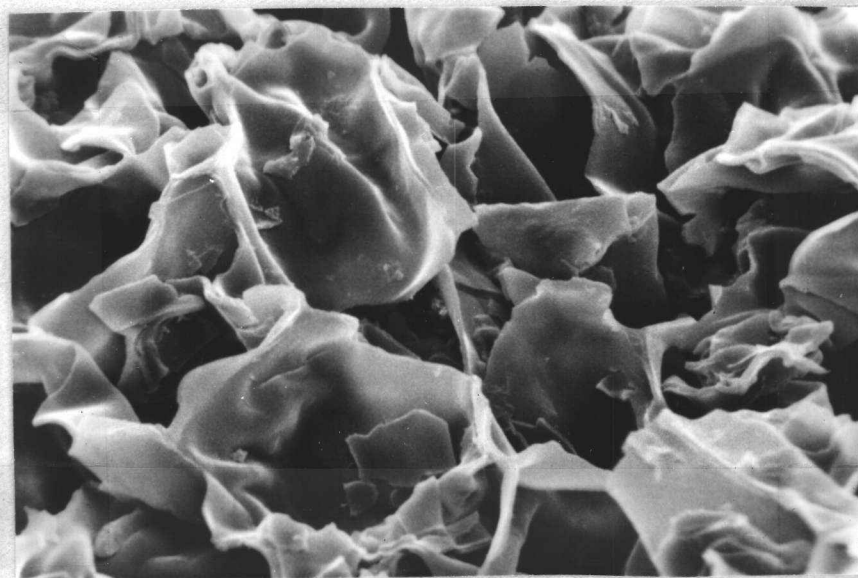
Figure 18A is of untreated cork and shows the brittle fractures across the cell wall produced by freezing the cork before grinding.

Figure 18. Scanning electron micrographs of the ground cork used in the chemical analysis.



Micrograph A - Unextracted cork

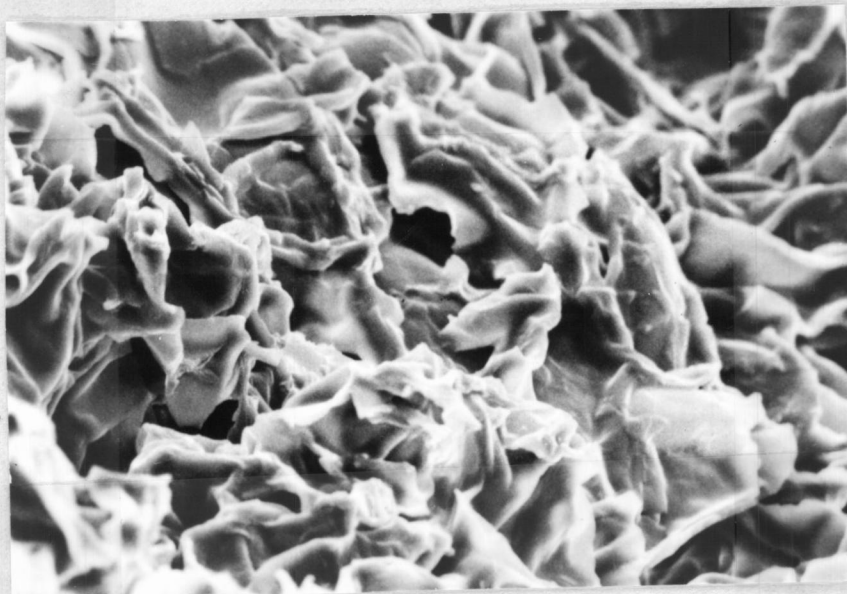
mag. 840X



Micrograph B - Benzene-ethanol, ethanol, hot water extracted cork

mag. 1182X

Figure 18. (Continued)



Micrograph C - Sulfite treated cork

mag. 840X



Micrograph D - After saponifying with 3% ethanolic potassium hydroxide for 8 hours

mag. 782X

Figure 18B when compared to 18A shows that the surfaces of the cork cells are clean and smooth after extraction showing no texture on the wall surface. Also the cell fragments seem to have collapsed upon each other. The same can be said for Figure 18C.

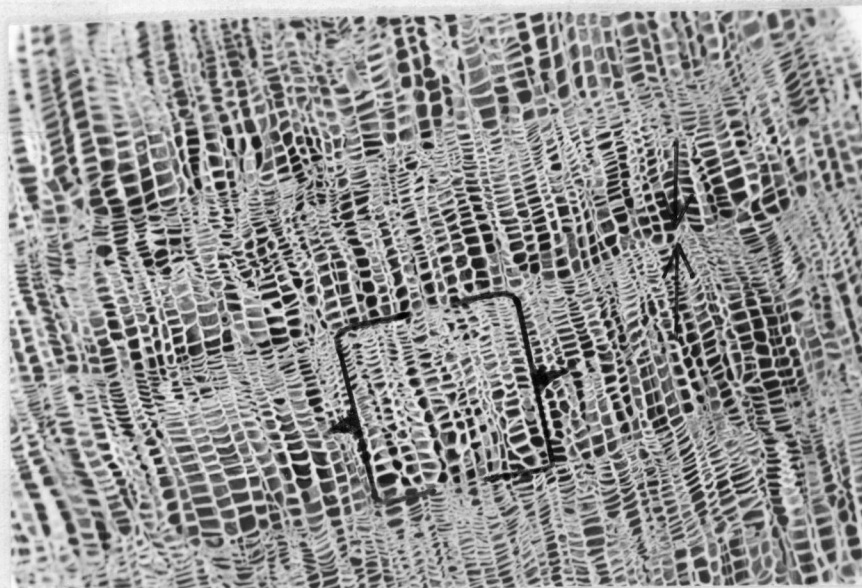
After saponification (either aqueous sodium hydroxide or ethanolic potassium hydroxide) changes can be seen to have occurred on the cell surfaces. Figure 18D is representative of these changes. In comparing 18D to 18A one should note the loss in recognition of the cell fragments. Also the cell surfaces appear very textured and look as if layers of material have been stripped from them. Also there is the hint of possible pits which appear as dimples and are indicated by the arrows in 18D.

Figures 19A-19G are of cork sections having chemical treatments comparable to those received by the ground cork. Figure 19A is a low magnification picture of untreated Douglas-fir cork showing the growth rings in a periderm (the bracket shows one growth ring). The arrow shows the point of transition between the crushed cells of one growth ring and the uncrushed cells of the adjacent growth ring.

Figure 19B is a higher magnification of this transition zone. The arrow shows the last formed cell wall of one growth ring and the first formed cell wall of the adjacent growth ring.

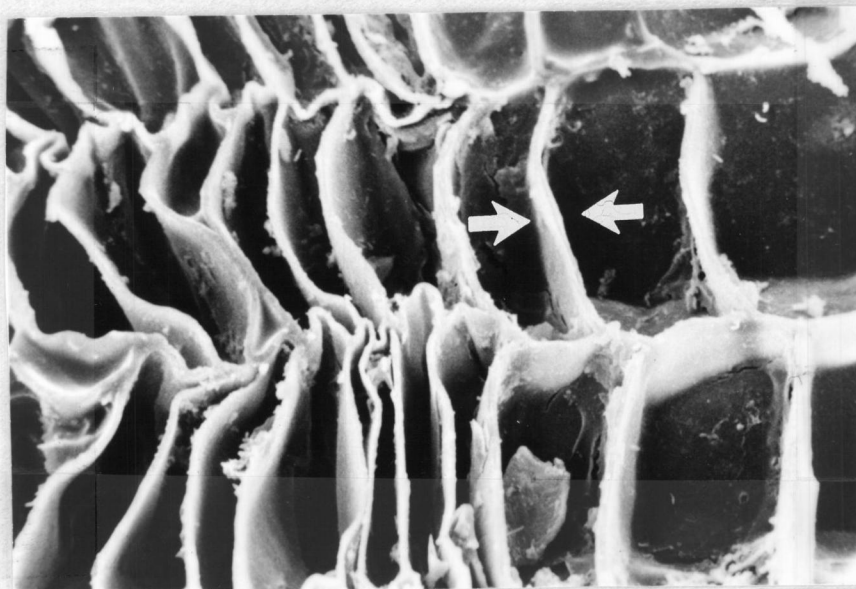
Figure 19C is of a cork section which has been extracted with benzene-ethanol and then ethanol. In comparing 19C to 19B one

Figure 19. Scanning electron micrographs of cork sections having various chemical treatments



Micrograph A - Unextracted cork - critical point dried

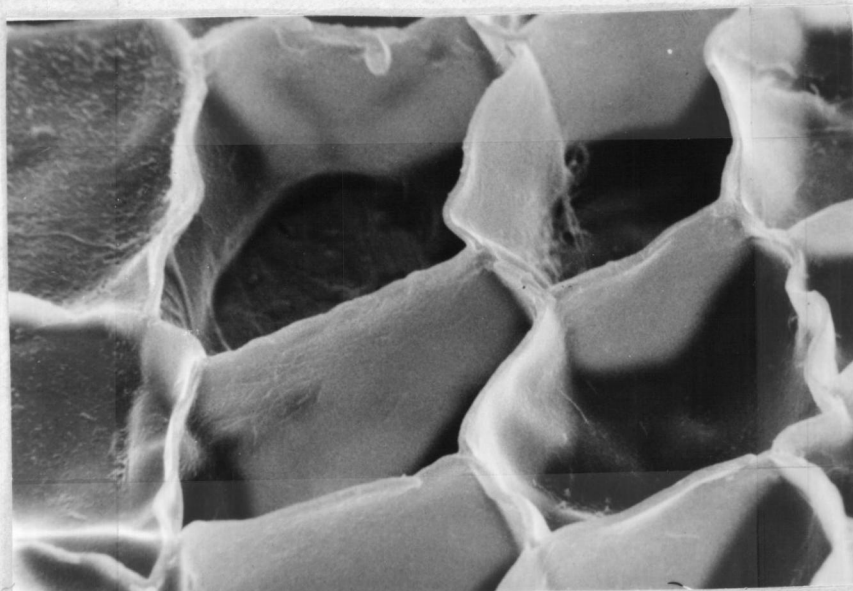
mag. 60X



Micrograph B - Unextracted cork - critical point dried

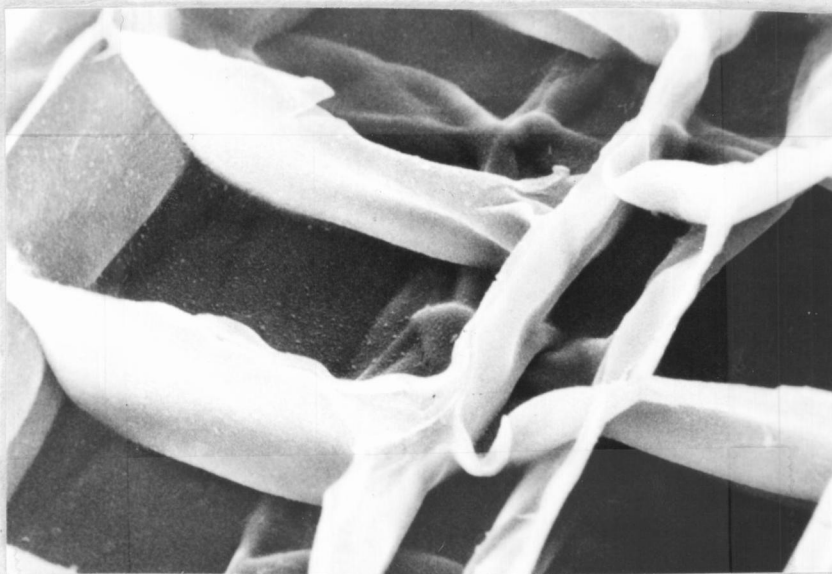
mag. 816 X

Figure 19. (Continued)



Micrograph C - Benzene-ethanol/ethanol extracted cork - acetone dried

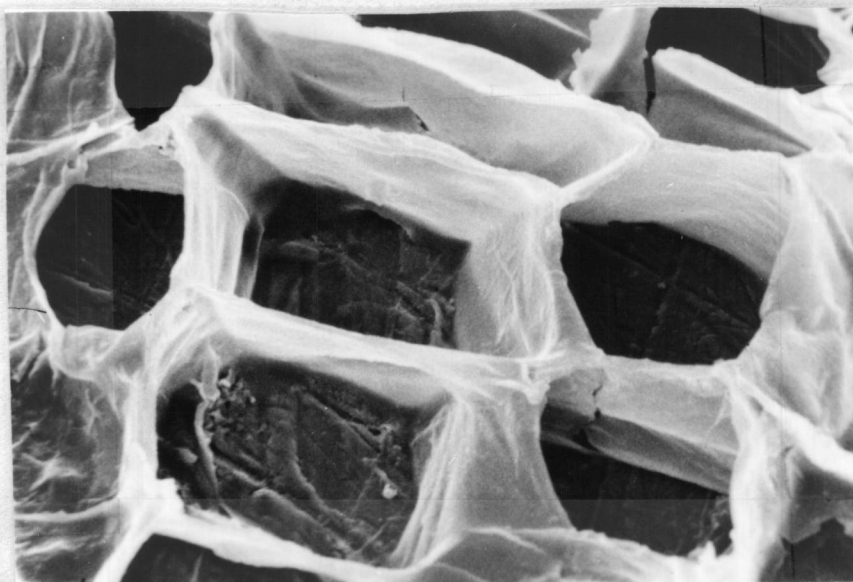
mag. 1151X



Micrograph D - Ammonium oxalate extracted cork - acetone dried

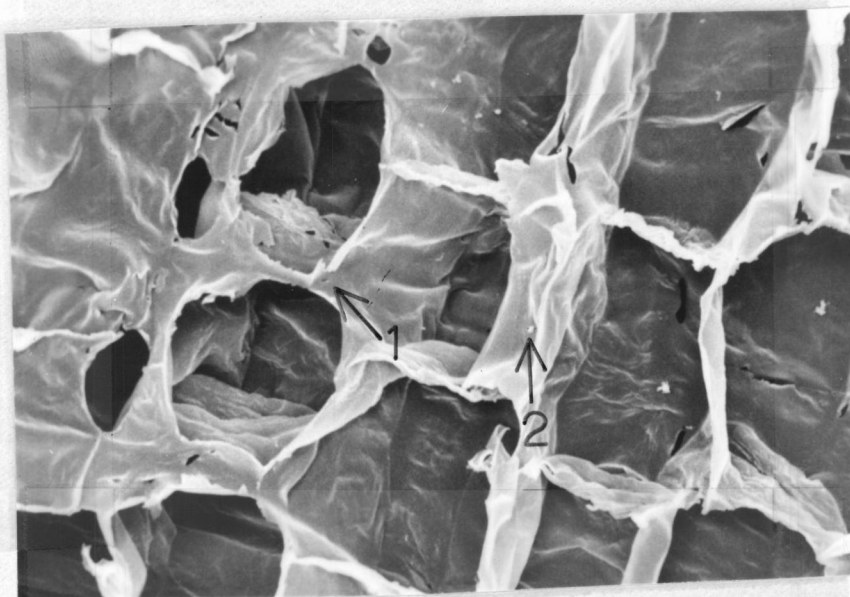
mag. 1154X

Figure 19. (Continued)



Micrograph E - Sulfite treated cork - acetone dried

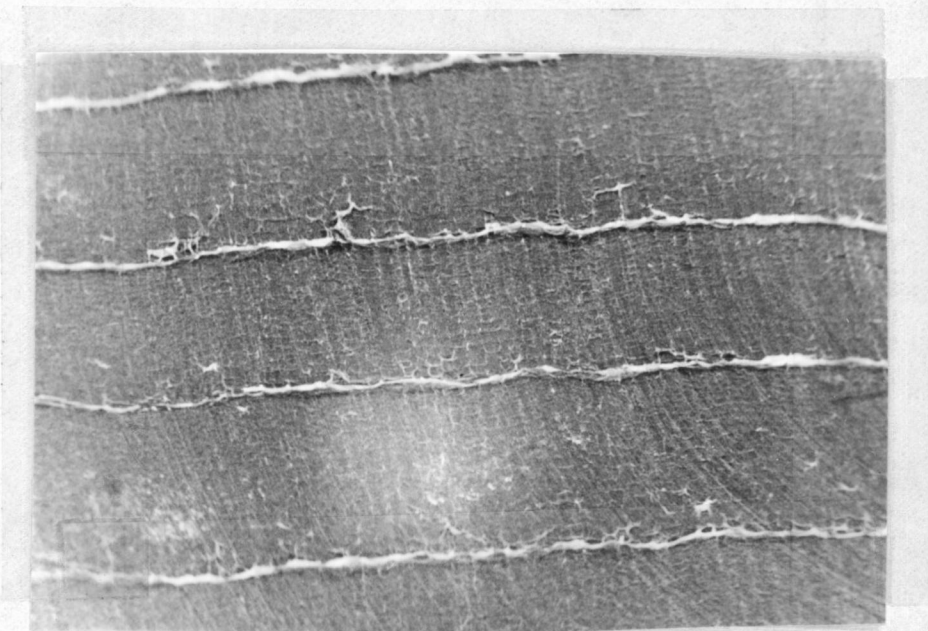
mag. 1182X



Micrograph F - Cork after treatment with aqueous sodium hydroxide - critical point dried

mag. 1151X

Figure 19. (Continued)



Micrograph G - Cork section after saponifying 8 hours in
aqueous sodium hydroxide - acetone dried

mag. 55X

should note, as indicated before with the ground cork, the smoothness and cleanliness of the cork cell walls.

Figure 19D is of a cork section treated with ammonium oxalate. No differences can be seen when comparing 19D to 19C.

Figure 19E is of a sulfite treated cork section. Unlike the previous sections, the cell walls in this section exhibit drying stresses, which appear as folds in the wall. Figures 19C, D, E and G were all dried on the mounting stubs from acetone; they were not critically point dried. In comparing 19E to 19D one can say that the walls of cork shown in Figure 19E are weaker than those of 19D. Thus the sulfite treatment has weakened the cork cell walls. Thickness changes in the cork cell walls cannot be monitored using the scanning electron microscope. Conclusions concerning possible causes are discussed under the results section on T. E. M. (p. 75) in this thesis.

Figure 19F is of cork after prolonged treatment with aqueous sodium hydroxide. The section was critically point dried and should not show any drying stresses. However, the cell walls are severely folded and appear to be much thinner. Most important to notice is 1) the appearance of small round holes in the wall and 2) the small white dots which appear to be deposits on the cell walls (both of the above are designated by arrows). I believe the above represents the pits of the cork cell and callose deposits which plug the pits in the

cork cell.

Figure 19G represents a cork section which, after 8 hours of refluxing in 3% aqueous sodium hydroxide, was dried from acetone onto a mounting stub. The white parallel lines running from left to right represent wall material which is very alkali resistant and is present in the first formed cell wall of a growth increment (see Figure 19B). This wall is different and must be examined independently of the other cell walls. Differences in this wall from the other cell walls are discussed under the results section on T. E. M. (p. 78).

Transmission Electron Microscopy

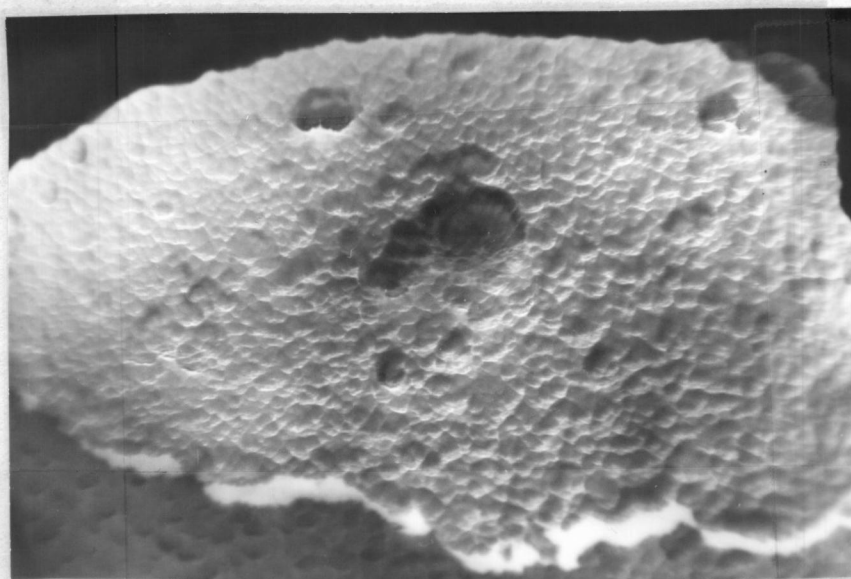
Replication

Replicas are a means of studying the ultra-structure of surfaces. This technique is capable of finer resolution than that achieved by the scanning electron microscope. When the two are used in conjunction with each other the researcher has at his disposal a full range of magnifications with acceptable resolution. The attainable resolution of replicas is approximately 30-100 Å while the scanning electron microscope can only achieve resolutions of approximately 150-200 Å. The results of the replica study are shown in Figures 20A-20D.

Figure 20A is of a replica from untreated cork which shows the very coarse texture of the wall surface on the lumen side.

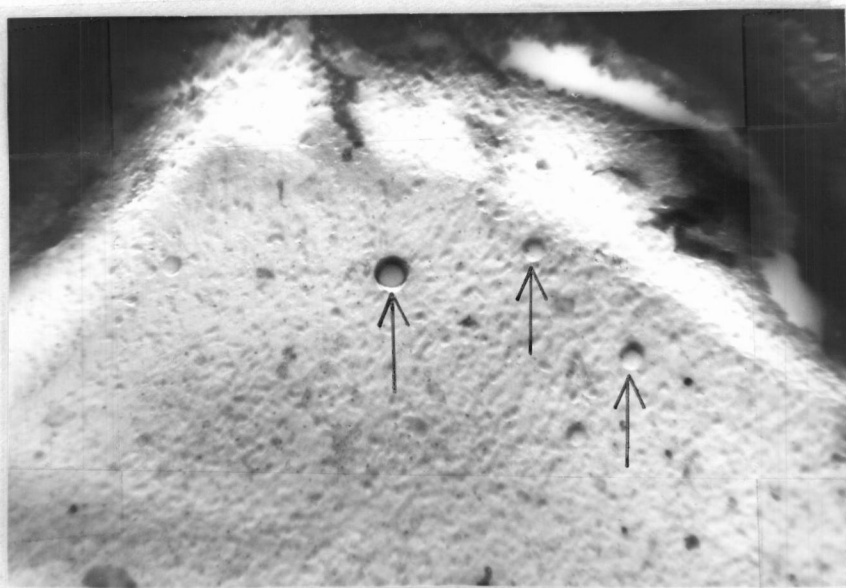
Figure 20B is of a replica from benzene-ethanol, ethanol

Figure 20. Transmission electron micrographs of cork replicas, representing various chemical treatments



Micrograph A - Untreated cork

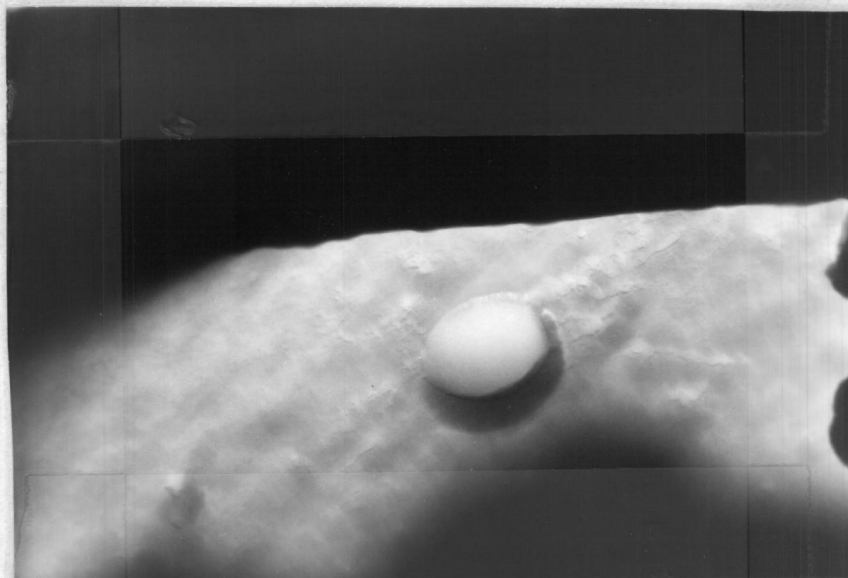
mag. 5317X



Micrograph B - Benzene-ethanol/ ethanol extracted cork

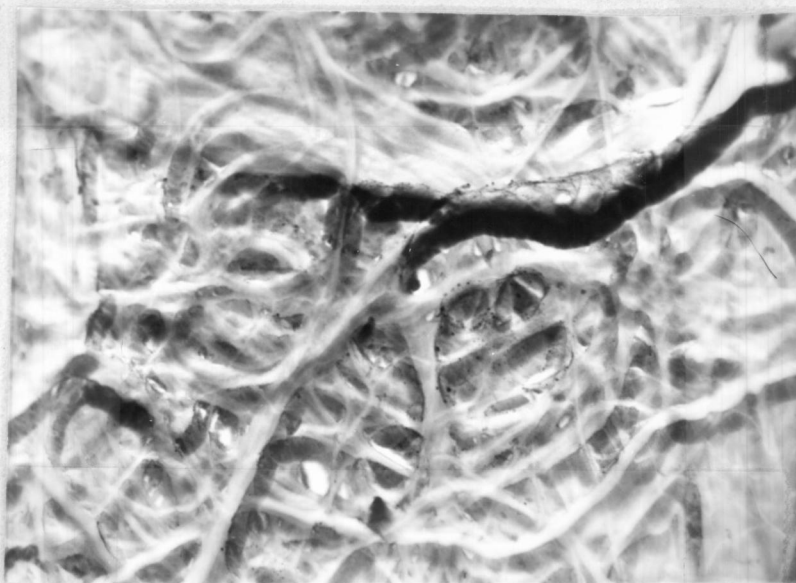
mag. 3866X

Figure 20. (Continued)



Micrograph C - Callose plug from benzene-ethanol/ethanol extracted cork

mag. 20, 209X



Micrograph D - Microfibrils from saponified cork

mag. 12,867X

extracted cork. In comparing 20B to 20A, the main differences in the surface texture of 20B is that it is less coarse than that of 20A. It is important to note that some texture is still exhibited by 20B. Further explanation of this point will be discussed under the discussion section of this thesis (p. 96).

I also believe one can see definitive callose plugs in 20B (designated by arrows). Figure 20C is a high magnification close up of a suspected definitive callose plug.

Figure 20D is a micrograph of a replica from cork treated with aqueous sodium hydroxide for a prolonged time. The random orientation of the microfibrils would indicate that the surface is the cellulosic primary wall. Microfibrils are visible only after the saponification reaction. This would be expected because the study on the saponification reaction showed that cork cells stained with the IKI solution only turned blue (indicating cellulose) after saponification. The size of the microfibrils shown in Figure 20D range from 300 \AA to 1000 \AA .

Sections

The analysis of embedded and sectioned material in the transmission electron microscope allows the researcher to observe the inner structures of this material. A significant and crucial facet in the use of this tool lies in the ability to enhance or selectively stain the areas of interest.

In this study six different staining techniques were used. The first was a pectin stain developed by McCready and Gee (12, 28) and used by Albersheim (1). This stain is selective in that it stains only methalated uronic acids.

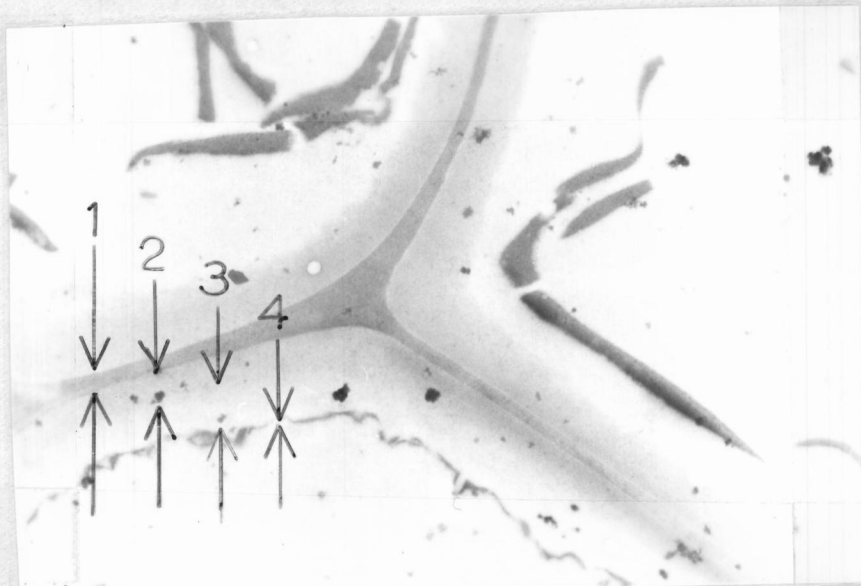
Three of the techniques used osmium stains, but they differed in the pretreatment given to the cork immediately before staining with osmium. Thus the staining patterns seen were the results of the pretreatment and not the osmium. The pretreatment was to soak the cork in acid, neutral or alkaline solutions covering a range of pH conditions before staining with osmium. The logic for using these pretreatments was that different chemical functional groups become available for staining at different pH's. The expected staining patterns from these three pretreatments were: 1) the neutral pretreatment would serve as a control and would be similar to the results obtained by Sitte (37), 2) the acid pretreatment would protenate the phenolic hydroxyls and the acidic pectins thus making these groups more receptive to the osmium stain, 3) the alkali pretreatment would deprotenate the phenolic hydroxyls and acidic pectins and would swell cellulose and break ester linkages. By comparing the acid to the alkaline pretreatment one might learn the location of acidic pectins and if the phenolic hydroxys were protenated or deprotenated in the cell wall.

The last two staining techniques also used osmium but once

again the end result was due to the pretreatment given to the cork. The techniques used were pioneered by Seligman (36) and are used for selectively staining areas high in carbohydrates. The cork received a periodate oxidation and then stained in osmium, or received a periodate oxidation followed by reaction with thiosemicarbazide and then stained with osmium. Comparison of the cork which received the thiosemicarbazide reaction to the cork which received only the periodate oxidation reveals areas of high carbohydrate concentration. One important fact is that for these techniques to work the carbohydrate must have adjacent hydroxyls, as in cellulose. Callose which is linked β -1 \rightarrow 3 will not react since it does not have adjacent hydroxyls. The results of this study on cork sections are shown in Figures 21-26.

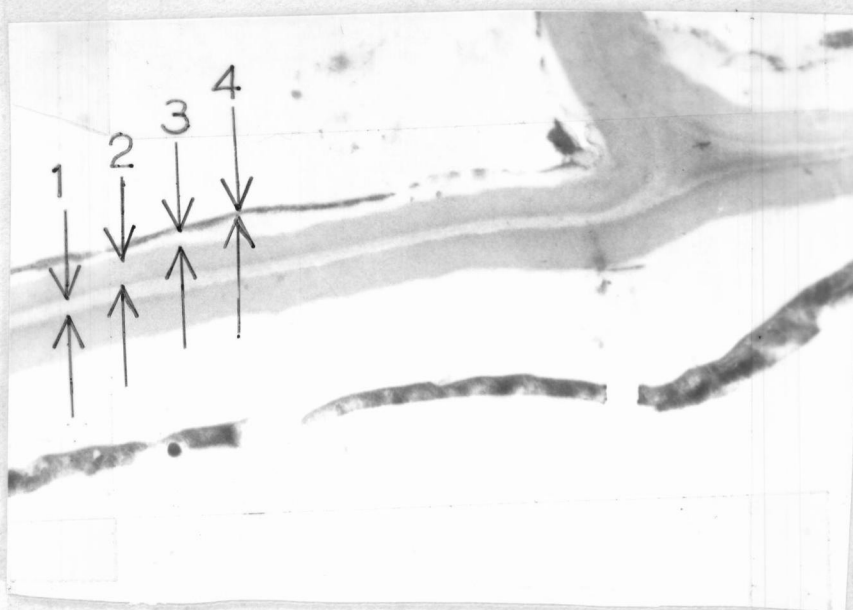
Figures 21A-21F are of radial sections of untreated cork and represent the different staining techniques used. In comparing the staining effects on the cork double walls shown, one should note the differences between the four zones which make up the double wall. Special attention should be given to the junctions where more than 2 cells meet. In Figure 21C I believe zone 1 is the compound middle lamella which is made up of the middle lamella and the primary wall. Zone 2 is the secondary layer made up of suberin. Zone 3 is the wax extractives and zone 4 is the old cell membrane, cytoplasm or other cellular debris. For convenience, zone 4 will be referred to as the

Figure 21. Transmission electron micrographs of unextracted cork sections showing differences in staining techniques



Micrograph A - Pectin stained

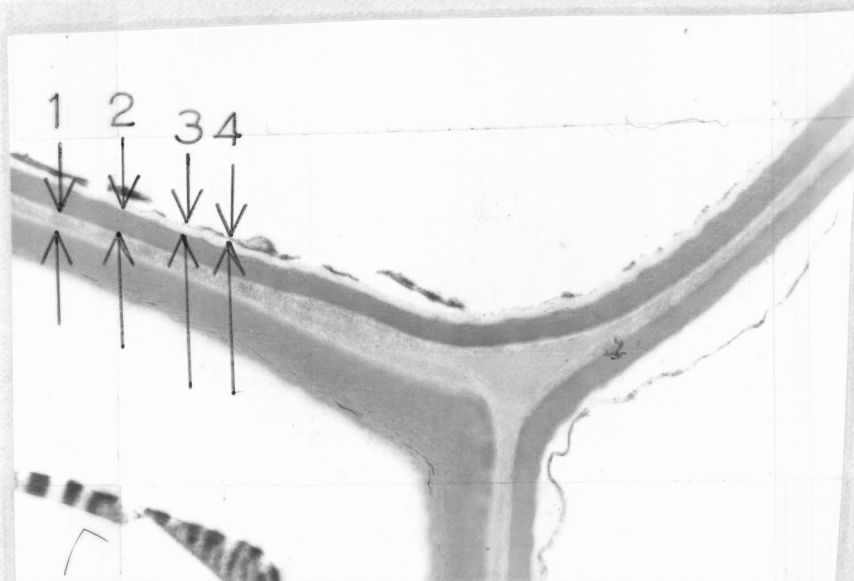
mag. 12, 250X



Micrograph B - Neutral pretreatment osmium stained

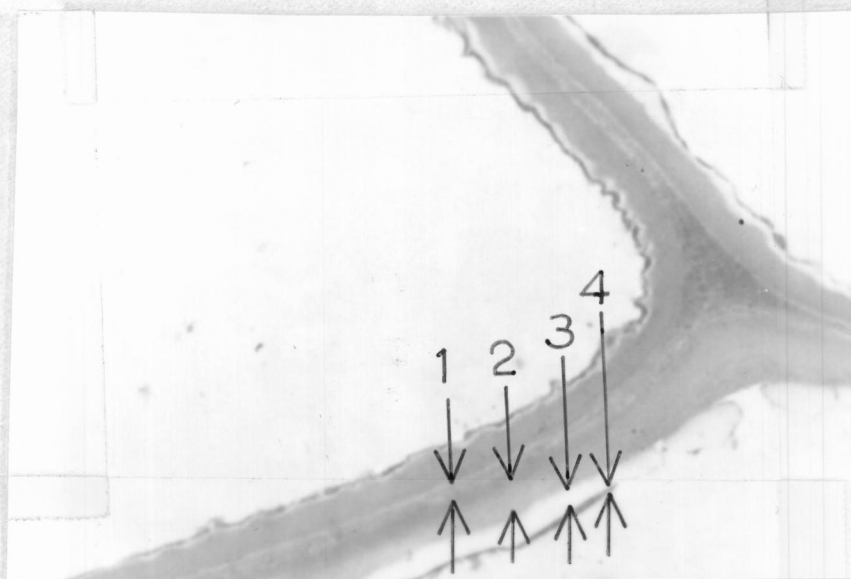
mag. 15, 500X

Figure 21. (Continued)



Micrograph C - Alkali pretreatment osmium stained

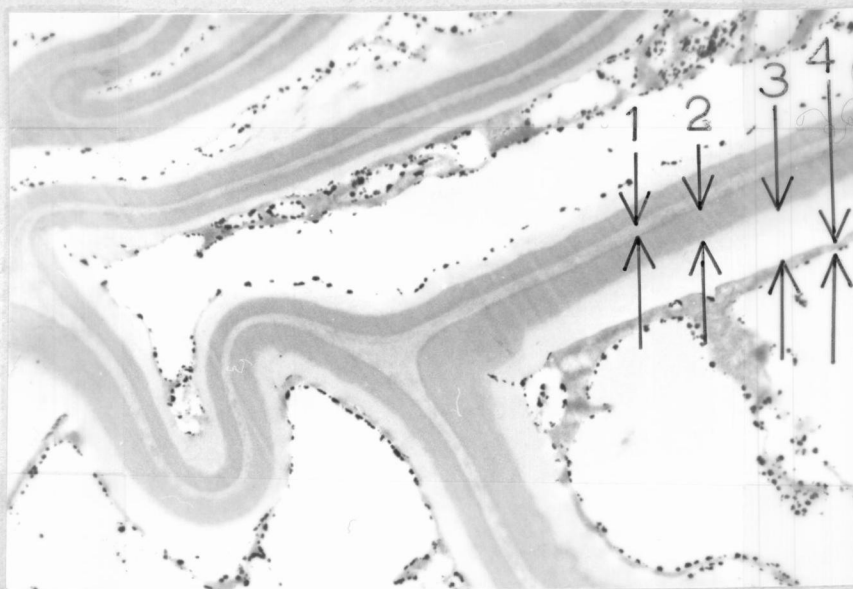
mag. 9,700X



Micrograph D - Acid pretreatment osmium stained

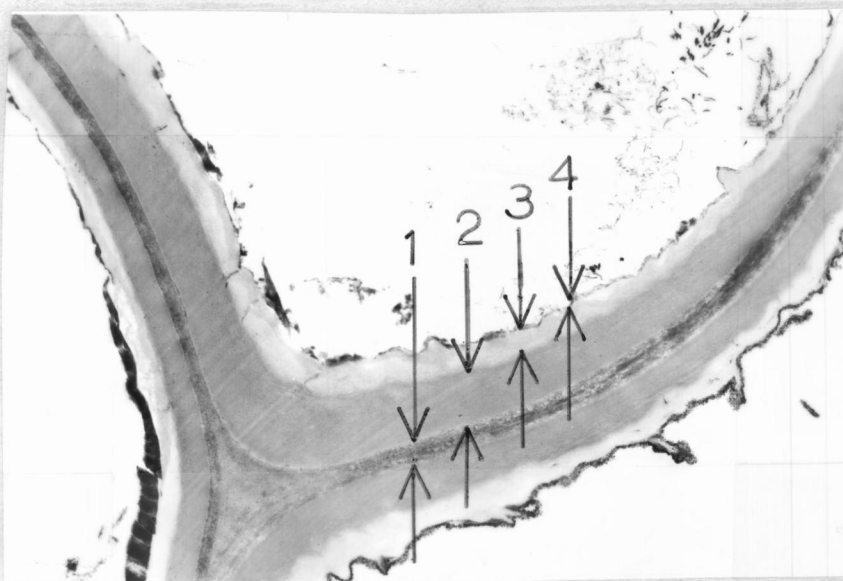
mag. 10,614X

Figure 21. (Continued)



Micrograph E - Periodate oxidation pretreatment osmium stained

mag. 12, 500X



Micrograph F - Periodate oxidation - thiosemicarbazide pretreatment osmium stained

mag. 10, 907X

"cytoplasm layer" but one should remember that the composition of this layer is more complex than cytoplasm.

Figure 21A shows the effect of the pectin stain. In zone 1 the middle lamella is much darker than the other 3 zones. These results are consistent with the fact that most pectin is found in the middle lamella.

In comparing zone 1 in Figure 21B-21D one should note that the neutral treatment (pH 7.0, Figure 21B) is somewhat inconsistent in that dark colored and light colored zones can be found. With the alkali treatment (pH 14, Figure 21C) the middle lamella appears lightly stained while the acid treatment (pH 2.0, Figure 21D) allows the middle lamella to stain more heavily. The suberin layer, the wax extractives and the cytoplasm do not change, or change little with these 3 staining techniques.

Figure 21E is of a cork section oxidized by periodate and stained with osmium. Figure 21F is of a cork section which after being oxidized with periodate was reacted with thiosemicarbazide and stained with osmium. These two figures, when used together, show where carbohydrates are localized. Those areas which are light in 21E but are dark in 21F are areas of high carbohydrate concentration. In comparing these two pictures the main differences are: 1) the middle lamella area of 21E is light while in 21F it is dark, 2) the primary wall has become dark in 21F, and 3) small

cracks or pores appear in the wax extractives zone of 21F (designated by arrows in Figure 21F). Thus it is believed that these areas contain a high concentration of carbohydrates.

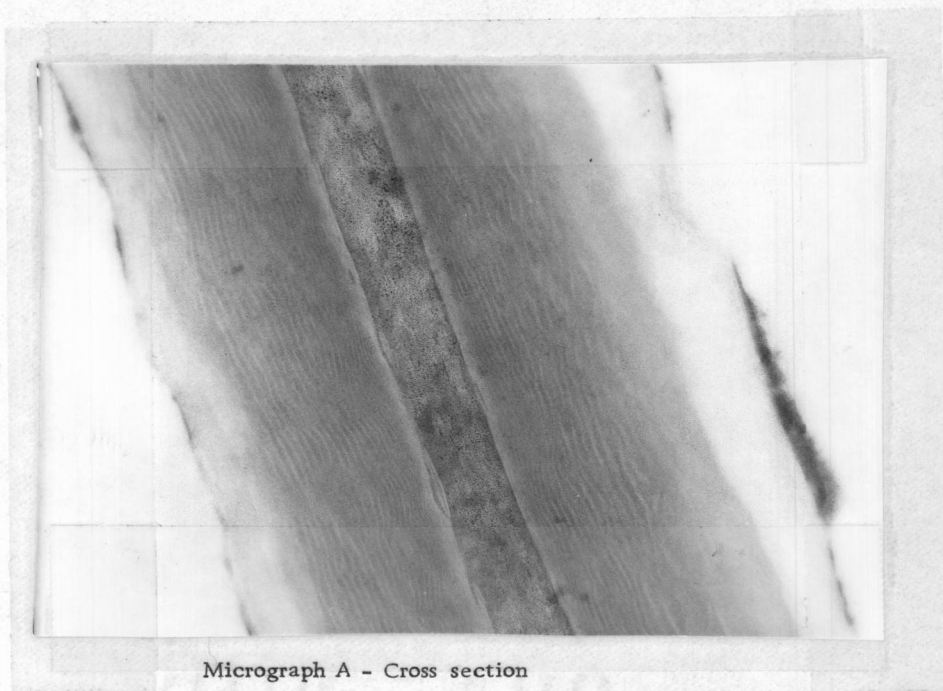
Figures 22A and 22B are of cross and radial sections stained comparable to that received by the cork in Figure 21D. The main things to be noticed on these pictures are: 1) the layered structure of the suberin zone, and 2) that this layering is the same on both the cross and radial sections. Therefore the layering must be composed of alternating lamellae of material and not rods or any other types of structures. Also to be noticed is that the layering is fine and uniform near the primary wall but as it extends inward toward the lumen the dark zones become thicker and the light lines tend to thicken and wave. This is most noticeable at the cell corners especially when there is a heavy deposit of the suberin (see Figure 22B area designated by arrows).

Figures 23A-23E show the effect that the various chemical treatments had on the cork cell wall.

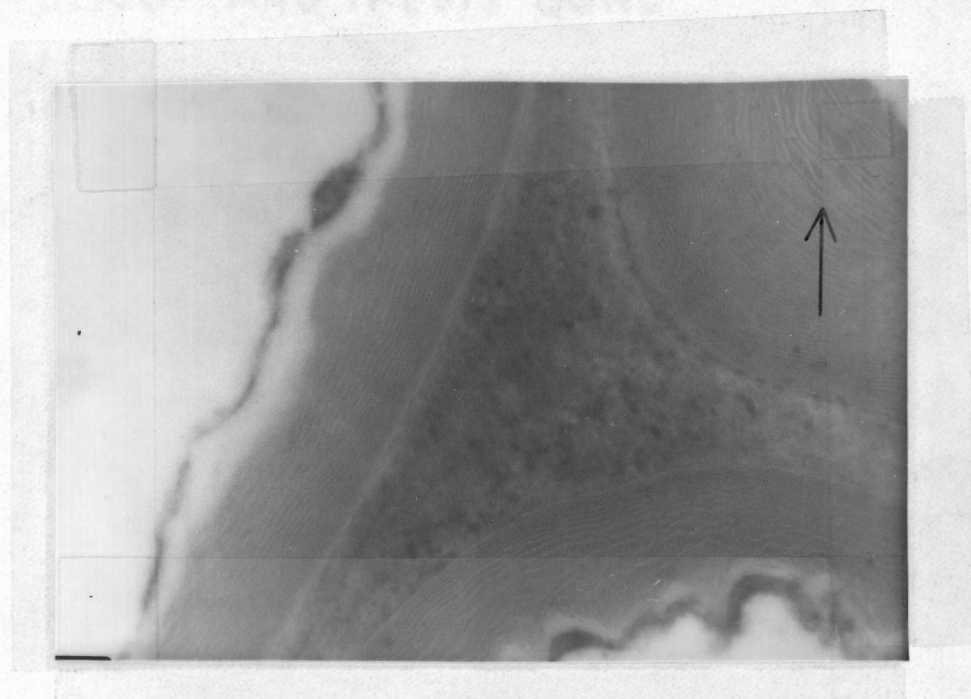
Figure 23A is of untreated cork. Because it has been stained with osmium after an acid pretreatment, the middle lamella shows up dark.

Figure 23B is of cork after extraction with the organic solvents.

Figure 22. Transmission electron micrographs of cross and radial sections of cork stained with osmium after an acid pretreatment

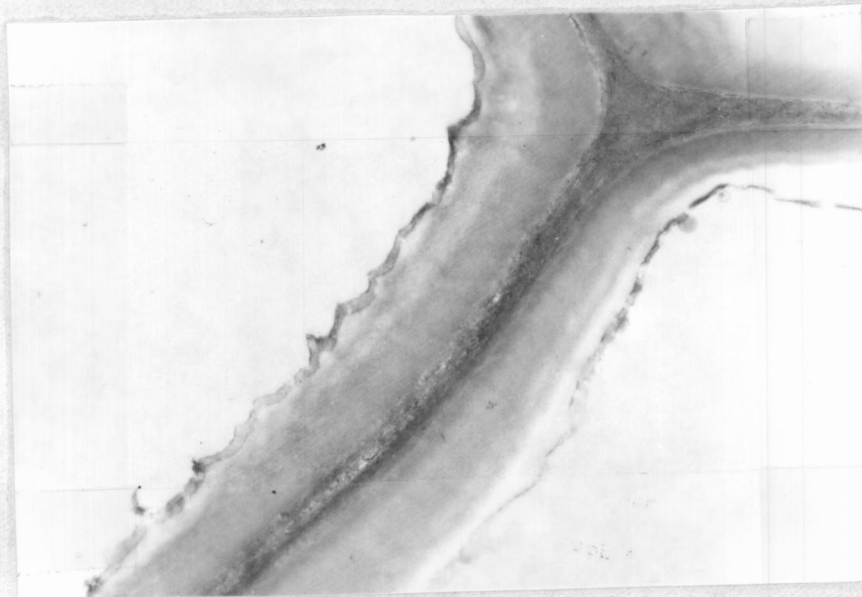


mag. 50,500X



mag. 48,809X

Figure 23. Transmission electron micrographs of radial sections of cork stained with osmium after acid pretreatment showing the effect of various chemical treatments on the cell wall



Micrograph A - Unextracted cork

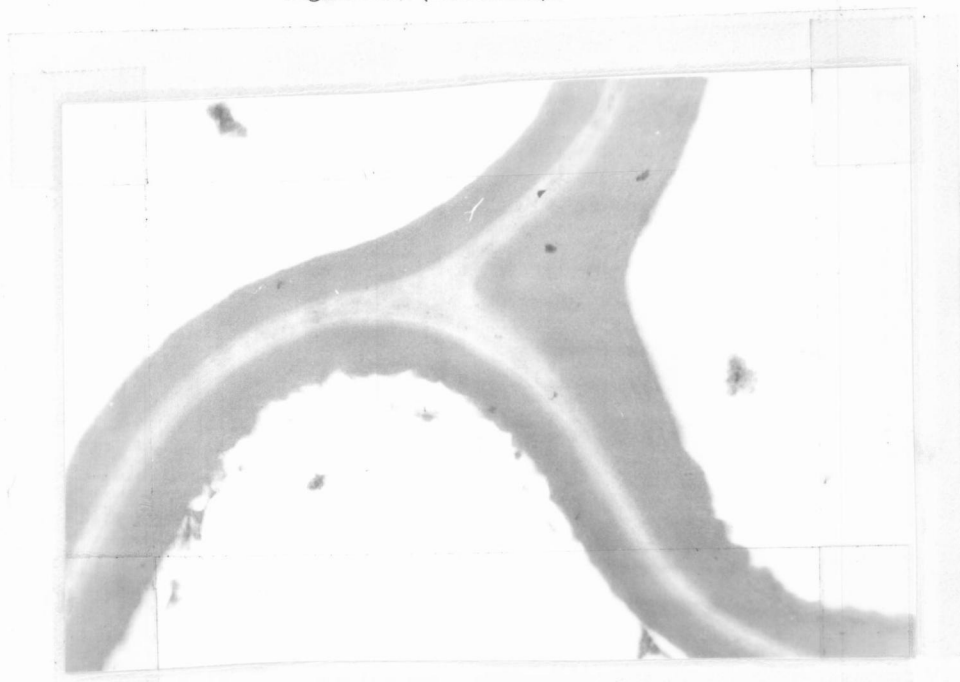
mag. 11,400X



Micrograph B - Benzene ethanol/ethanol extracted cork

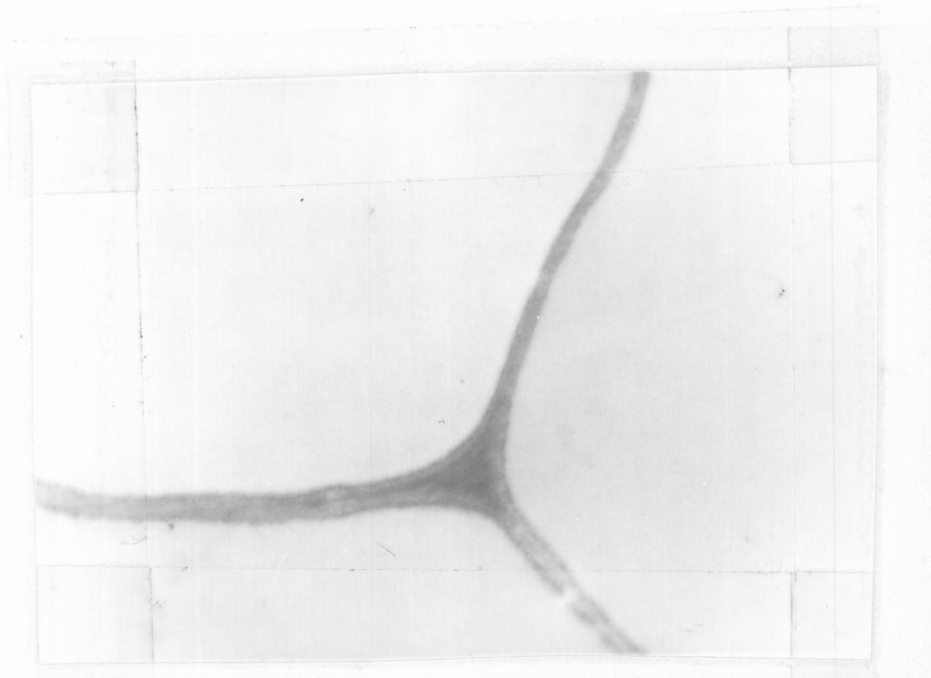
mag. 11,053X

Figure 23. (Continued)



Micrograph C - Hot water extracted cork

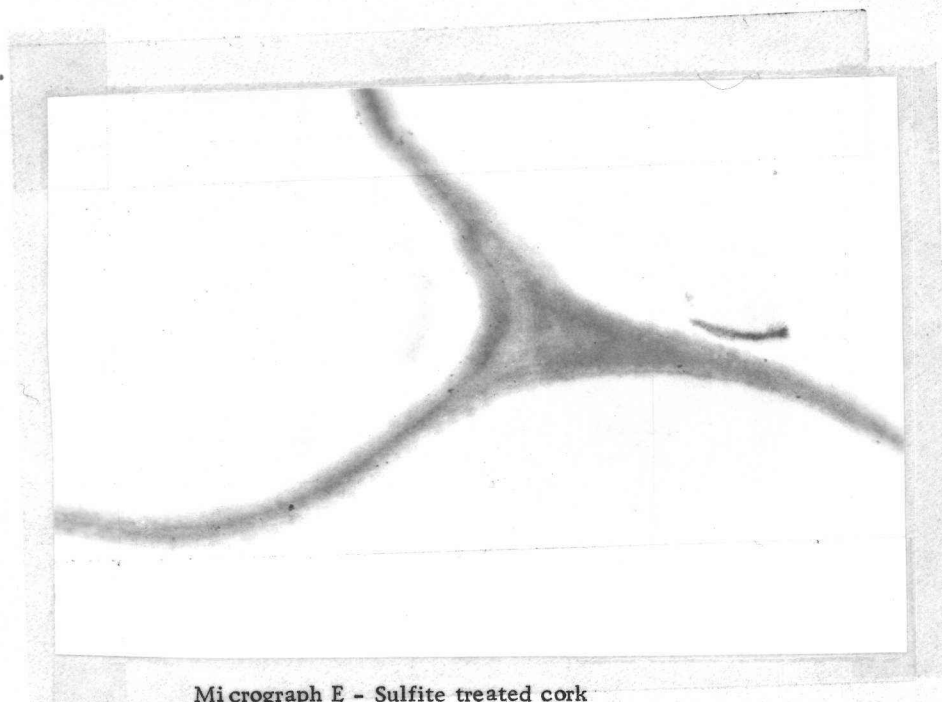
mag. 10,500X



Micrograph D - Cork after being saponified with 3% aqueous sodium hydroxide for 8 hours

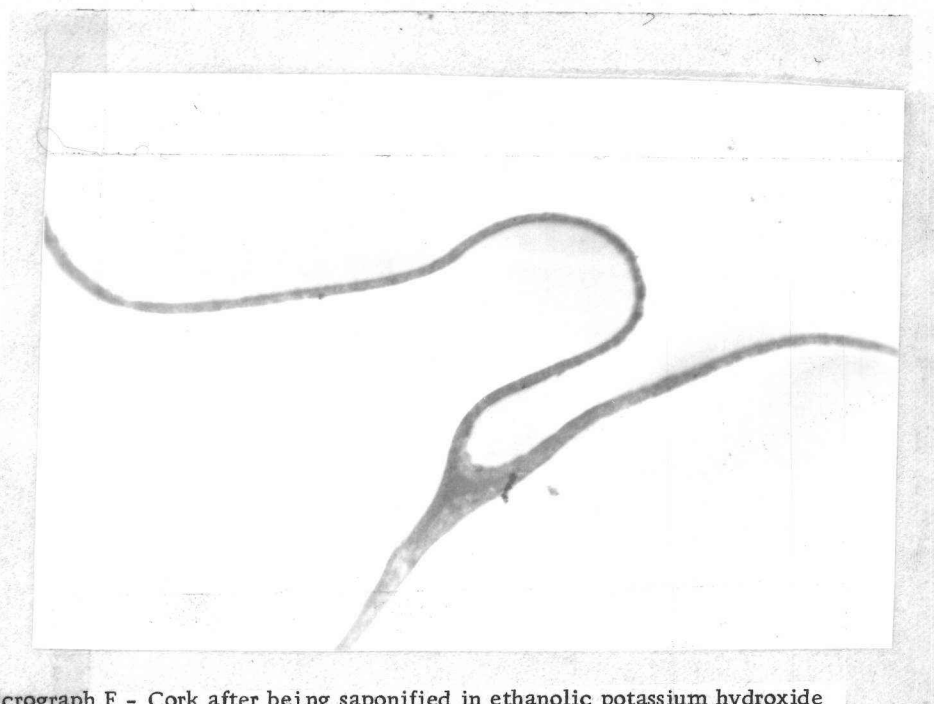
mag. 11,735X

Figure 23. (Continued)



Micrograph E - Sulfite treated cork

mag. 18, 240X



Micrograph F - Cork after being saponified in ethanolic potassium hydroxide

mag. 12, 667X

Zones 3 and 4 (see Figure 21C) have been lost in the extraction, but the middle lamella still stains dark. The double cell wall thickness which was approximately $2\text{ }\mu\text{m}$ is now approximately $1.2\text{ }\mu\text{m}$ thick.

Figure 23C is of cork after extraction with hot water. There is no decrease in double wall thickness, however, the middle lamella does not stain as dark as before, especially at the junction where the three cells meet. This loss of staining intensity in the middle lamella is most likely due to a loss of pectin materials with the hot water extraction.

Figure 23D is of cork after saponification for 8 hours in 3% aqueous sodium hydroxide. The most important thing to notice is the loss of the suberin layer. The double wall thickness is now approximately $.2\text{ }\mu\text{m}$.

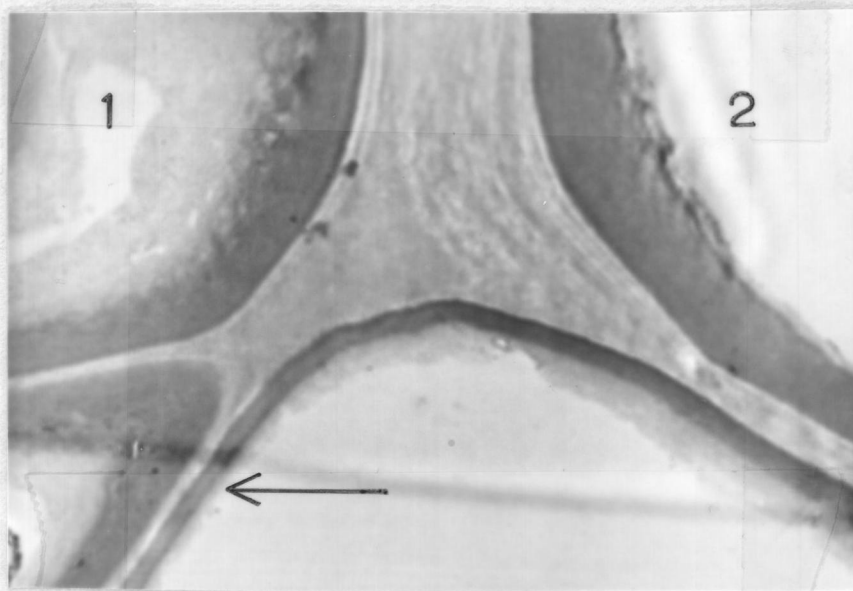
Figure 23E is of sulfite treated cork. Once again the most important thing to notice is the loss of the suberin layer. Although unexpected, this tends to support the scanning electron micrographs which showed a loss in cell wall strength with the sulfite treatment (Figure 19E). There is consistency between these observed facts and the chemical analysis for nitrogen loss. Large amounts of nitrogen were lost after the sulfite treatment and after the aqueous sodium hydroxide saponification reaction. This may indicate that large amounts of protein are present in the suberin layer.

Figure 23F is of cork after being refluxed in ethanolic

potassium hydroxide. Figures 23D and 23F are very comparable and one would expect the inner surfaces of the cell wall to be the same. However the results of the study on the saponification reaction show that after sulfite treatment only those sections treated with aqueous alkali stained blue, indicating the presence of cellulose. Those sections receiving an ethanolic alkali treatment stained brown. The interpretation of these results lead to the conclusion that the ethanolic solvent did not allow adequate removal of the suberin to take place and allow the cellulose in the primary wall to become exposed. Since the cork cell walls in Figure 23F appear to have all the suberin removed, a discrepancy with regard to the above conclusions seems apparent. There are two possible explanations: 1) a fine layer of suberin or some other material still remains on the wall to block the color reaction of the cellulose or 2) the ethanolic solvent reacts with the cellulose in some way to block the color reaction. To determine which of these was correct the following was done. Cork sections which were saponified with aqueous sodium hydroxide, stained with IKI and turned blue were put into a 3% ethanolic potassium hydroxide solution and refluxed for 1 hour. The sections were removed, washed and restained. The sections did not turn blue but stained brown. Thus it was concluded that alkaline ethanol does not allow the color reaction to develop even though the alkali and ethanol are supposedly washed out of the cell wall.

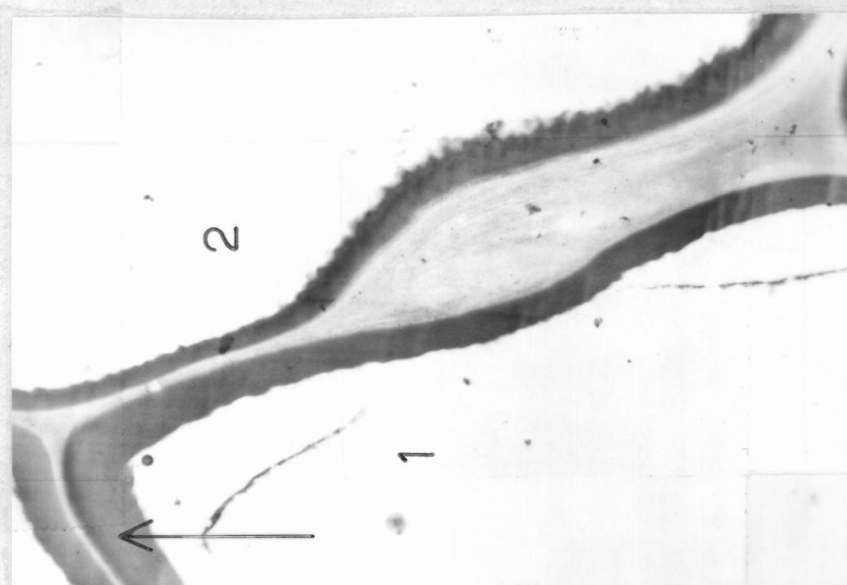
Figures 24A and 24B are of radial sections of cork at the

Figure 24. Transmission electron micrographs of radial sections of cork showing variations in wall thickness



Micrograph A - Unextracted cork having had an acid pretreatment before being stained with osmium

mag. 9,920X



Micrograph B - Extracted cork having had an acid pretreatment before being stained with osmium

mag. 6,500X

junction of the last formed crushed cork cell in a growth ring (designated #1) and the first formed uncrushed cork cell of the adjacent growth ring (designated #2). From these pictures it can be seen that: 1) the increased thickening is due to an increase in the compound middle lamella; 2) the increased thickness is restricted to one side of the cork cell (thickening occurs only on the tangential wall on the outer bark side of the cork cell; all other sides of the cork cell have normal walls). Also the increased thickness does not always extend the full height of the tangential wall but sometimes ends abruptly as shown in Figure 24B.

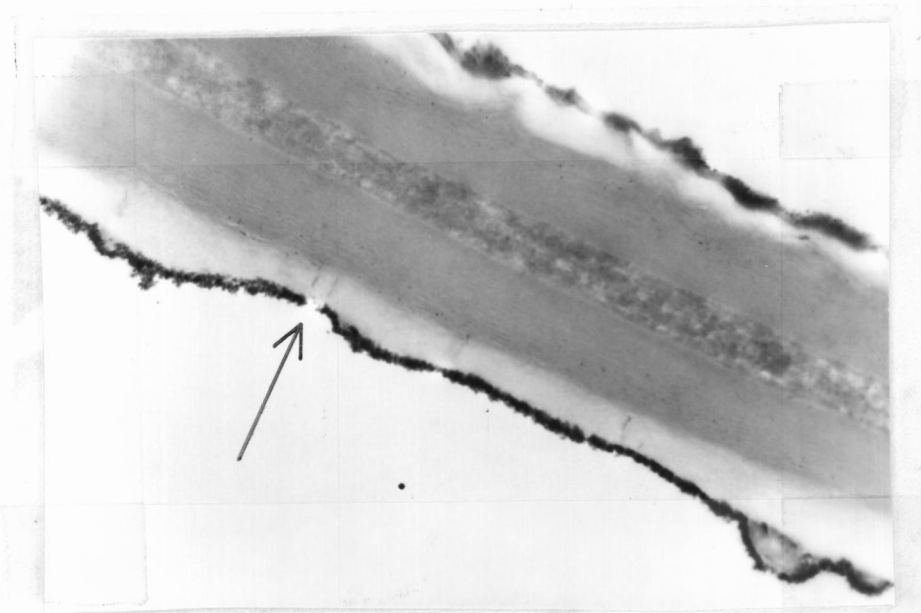
One last thing to be noticed in Figure 24A is the thick layer of extractives which has been built up in last formed crushed cell (#1) as compared to the lack of extractives in the first formed uncrushed cell (#2).

Figures 25A-25D are micrographs giving evidence for the existence of callose in the pits of the cork cell.

Figure 25A is a radial section of untreated cork stained with osmium after pretreatment with periodate and followed by thiosemicarbazide. The arrows in 25A denote possible pit locations.

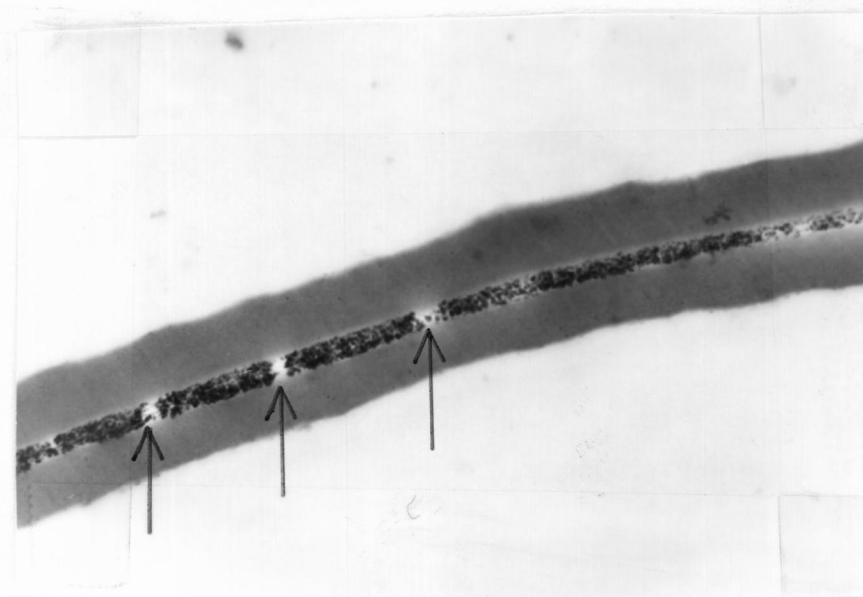
Figure 25B is of cork which has been extracted through the hot water treatment. This section was also stained similarly to that of Figure 25A. The arrows in 25B denote places where I believe callose plugs are present. The reasoning is that periodate will cleave β -1 \rightarrow 4

Figure 25. Transmission electron micrographs of radial sections of cork showing the existence of callose and the type of pitting present in the cork cell wall



Micrograph A - Unextracted cork pretreated with periodate and thiosemicarbazide before being stained with osmium

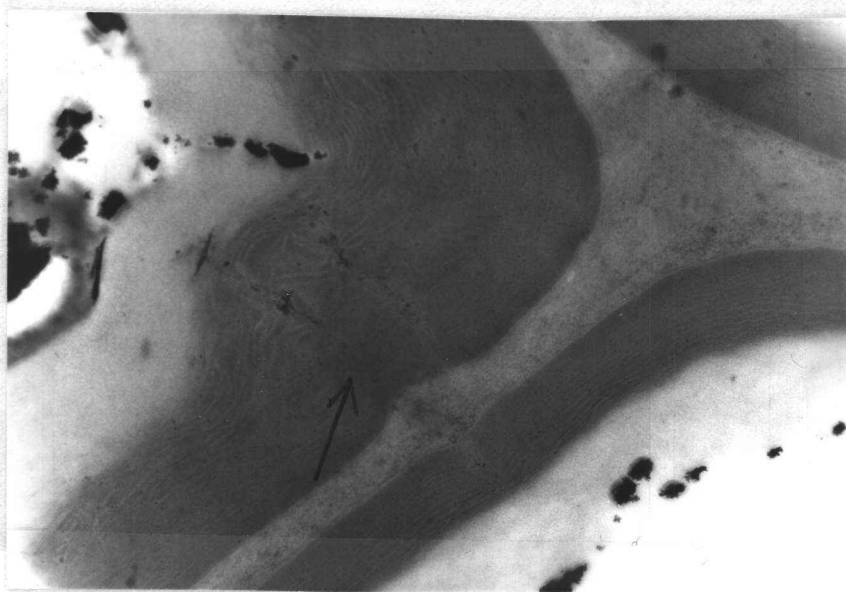
mag. 39,060X



Micrograph B - Hot water extracted cork pretreated with periodate and thiosemicarbazide before being stained with osmium

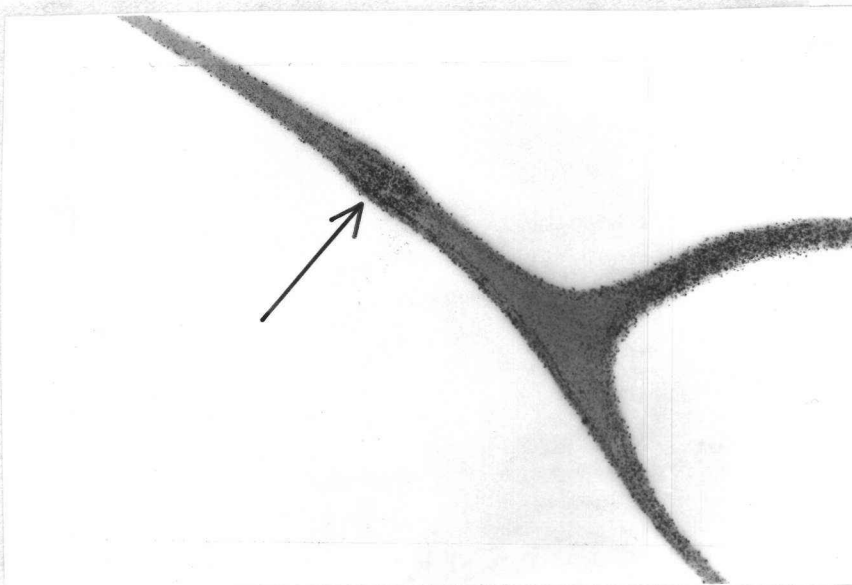
mag. 31,232X

Figure 25. (Continued)



Micrograph C - Unextracted cork pretreated with periodate and thiosemicarbazide before being stained with osmium

mag. 59,851X



Micrograph D - Cork after being saponified with 3% aqueous sodium hydroxide for 8 hours. Cork was pretreated with periodate and thiosemicarbazide before being stained with osmium

mag. 18,525X

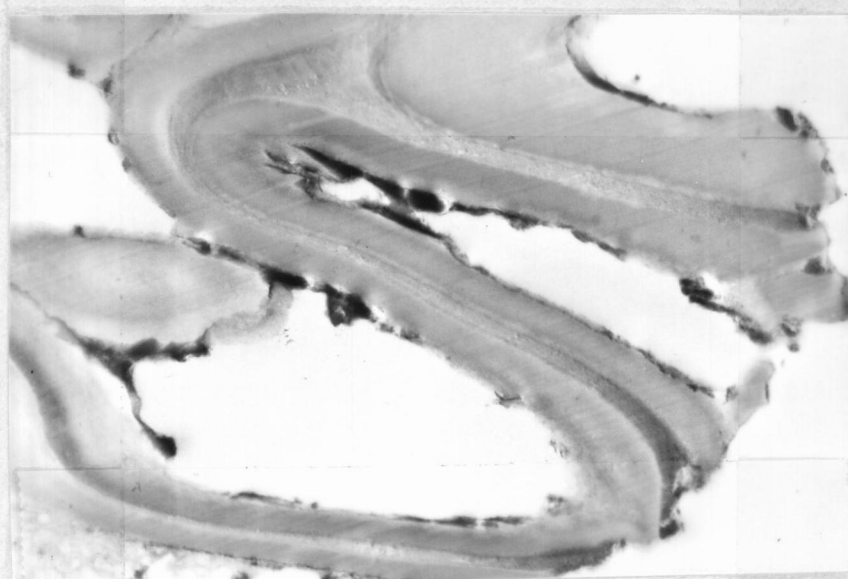
linkages and not β -1 \rightarrow 3, thus cellulose will stain and callose will not. Note in Figure 25B the very intense staining of the middle lamella as compared to 25A. I believe this is caused by better accessibility for the osmium in Figure 25B due to the extraction of the waxes and pectins.

Figure 25C is of an unextracted cork section which shows the callose plug extending from the middle lamella region through the suberin layer and ending at the start of zone 3 (arrows). The pit may extend through the wax extractives but this part of the pit does not seem to contain callose. The circumference of the pit from the middle lamella through the wax extractives seems to be lined with a carbohydrate. I believe the carbohydrate to be cellulosic and not pectic. The reasoning for this comes from Figure 25D.

Figure 25D is of a cork section after 8 hours of saponification in 3% aqueous sodium hydroxide. This section was stained with osmium after pretreatment with periodate and thiosemicarbazide. The micrograph shows the outline of the primary wall and a very dark stained bulge (denoted by an arrow). The dark stained bulge indicates that cellulosic material is present since pectic material should be removed by now. I believe the bulge represents the outer edge of the pit and not the material in the pit.

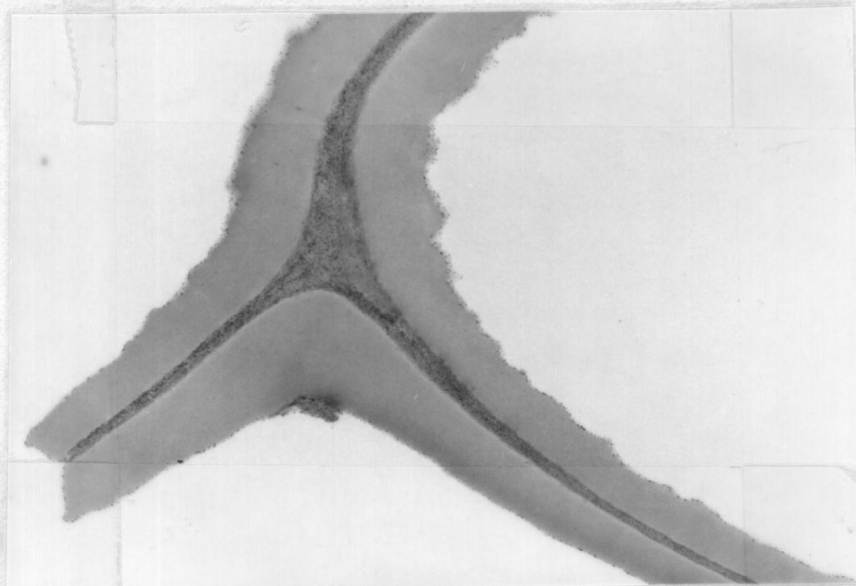
Figures 26A and 26B are of the ground cork used in the chemical analysis. Picture 26A represents the ground unextracted cork.

Figure 26. Transmission electron micrographs of the ground cork used for chemical analysis



Micrograph A - Unextracted cork

mag. 8,846X



Micrograph B - Ammonium oxalate extracted cork

mag. 16,477X

Picture 26B represents the ground cork which had been extracted with ammonium oxalate. These pictures confirm the fact that the fractures, produced by freezing the cork in liquid nitrogen and then grinding, were across the walls. They also show that the ground cork and the cork sections appear comparable after like chemical treatment. Thus it is valid to assume that the chemical treatments were not discriminatory and had the same effect on both ground cork and cork sections.

DISCUSSION

The first objective of this study was to determine the layering of the cell wall for the Douglas-fir phellem cell. Figure 27 is the proposed model of the wall showing the number of layers present and the chemical composition of each layer. It also represents an average between the extremes found in a growth ring. The first formed cell in a growth ring would have a tangential wall containing a thickened compound middle lamella and would lack a thick layer of wax extractives. The last formed crushed cells would differ from the model in that they would have a much thicker layer of wax extractives.

The above proposed model and Sitte's model of a cork cell from Quercus suber (39) are very much alike with regard to cell wall dimensions. Table 4 gives a comparison of the thickness of cell wall layering values obtained by Sitte and those I obtained using Figure 20A as the source of measurements. It can be seen from Table 4 that the values for the compound middle lamella, suberin and tertiary or cytoplasm layer are very comparable. The values for the thinnest wax lamella found in the suberin layer are very similar, as well as the values for the pit diameters. The only major difference is that Sitte's model does not have a wax extractive layer.

The chemical composition of each layer in my model is basically

Figure 27. Proposed model for the cell wall of a Douglas-fir phellem cell.

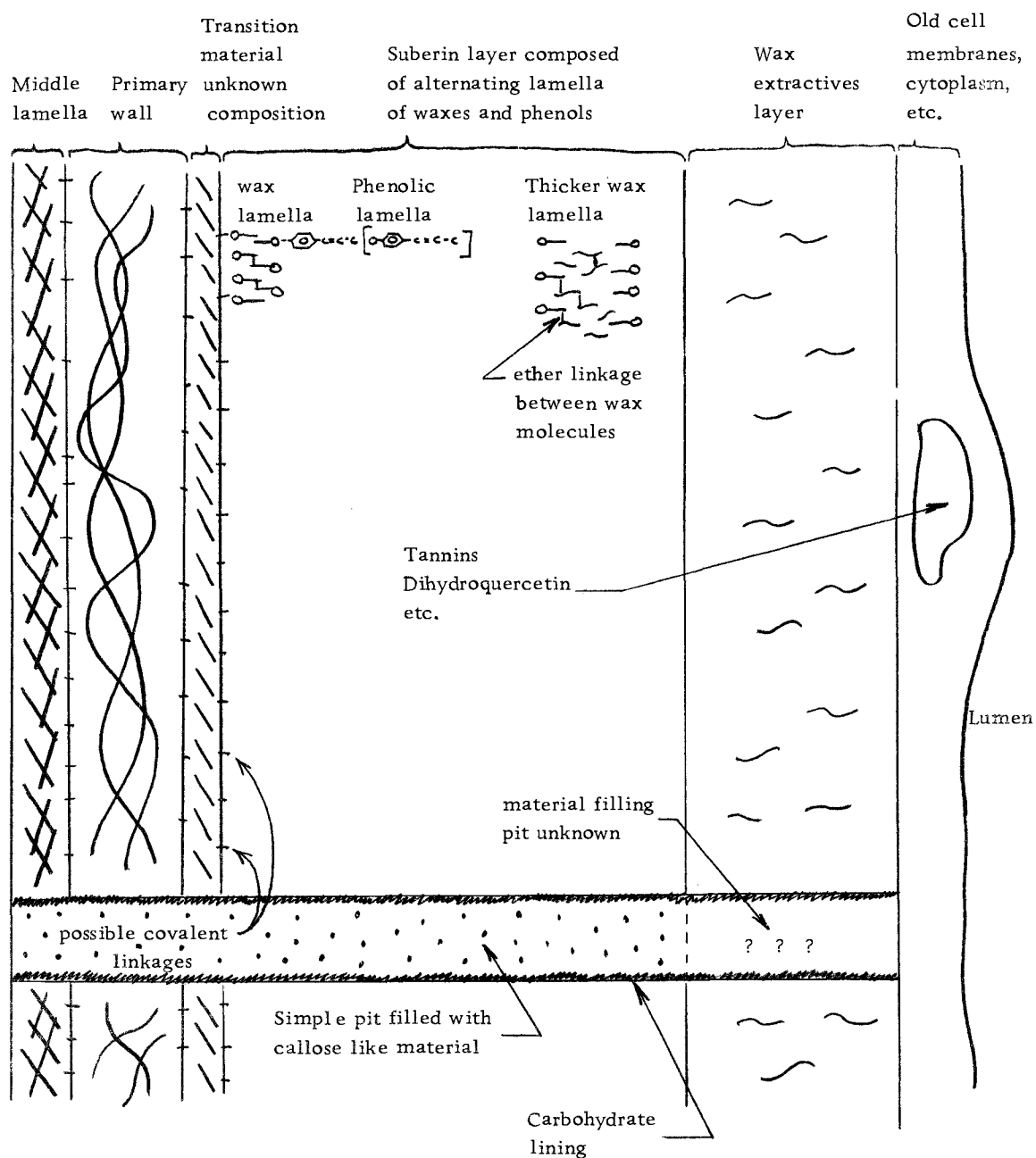


Table 4. Values for the thickness of layers found in the cork cell walls of Quercus suber and Pseudotsuga menziesii (Mirb.) Franco

Layer	Quercus suber according to Sitte values in nm	Pseudotsuga menziesii (Mirb.) Franco by Litvay values in nm
Compound middel lamella	115	198
Suberin	476	356
Wax extractives		300
Tertiary or cytoplasm layer	53 (120 max.)	46
Thinnest wax lamella in suberin layer	3	< 4 to > 200
Pits or pores (diameter)	60	100

the same as Sitte's; however, some major differences do occur, and therefore an explanation of each wall layer will follow.

The cytoplasm layer is not an artifact since it is continuous and appears on the lumen surface of all cells regardless of staining technique. Since the cytoplasm was removed with the wax extractives by the organic solvent extraction, the cork cells cytoplasm chemical makeup cannot be defined precisely. This layer reacts strongly with the electron microscopy stains unlike the wax extractives layer, therefore their compositions differ. Since the cytoplasm reacts it must be composed of chemicals which have reactive groups. In analysing the organic solvent extractives, chemical compounds which are saturated or contain few functional groups would belong to the wax extractives layer while those compounds which have reactive functional groups would most likely belong to the cytoplasm layer.

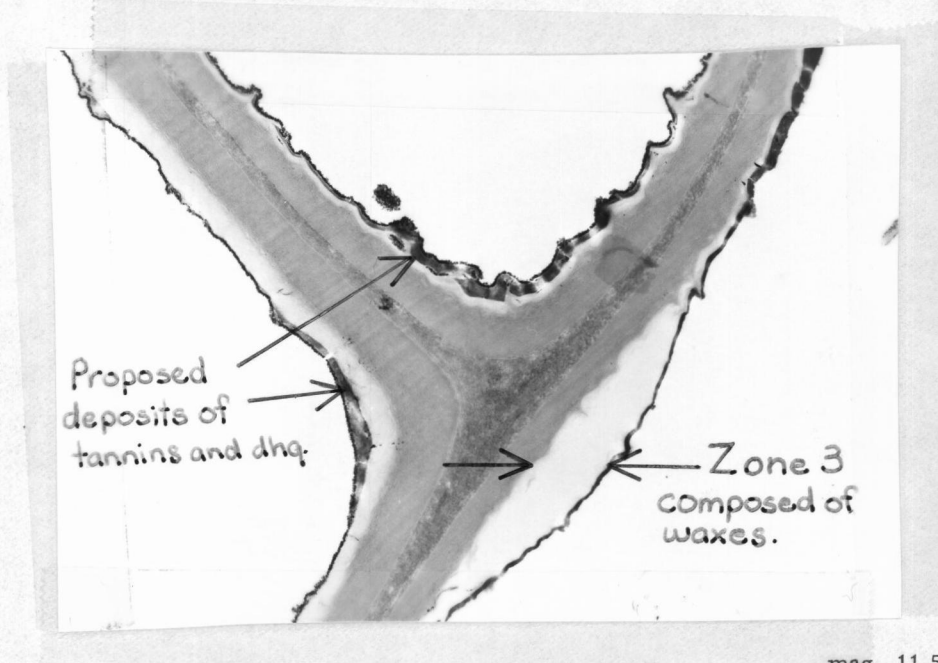
The analysis of the water soluble fraction of the organic solvent extractives revealed the fact that there were carbohydrates and phenols present. There was also a drop in nitrogen (or protein) content with organic solvent extraction. Since all of these chemicals possesses reactive functional groups they most likely help make up the cytoplasm layer. The cytoplasm and other membranes in plant cells have been shown to be composed mainly of proteins and lipids (30). A possible means of checking to see if this were also true of the phellem cell would be to do a hot water extraction before the

organic solvent extraction. This would strip off the cytoplasm layer and leave the wax layer intact. Quantitative and qualitative analysis of the water extract could then determine the amount and type of chemicals that make up the cytoplasm layer.

Since replicas of the surface of untreated cork did not reveal the presence of microfibrils nor did the light microscopy stain (IKI) show the presence of a cellulosic wall, it is unlikely that the cytoplasm layer is cellulosic and truly a wall layer. The cytoplasm layer is more likely membranes and cytoplasm which were deposited on the lumen surface when the cell died.

According to Kurth (24) the extractives of cork are composed of waxes, tannins, and dihydroquercitin. Tannins and dihydroquercitin are compounds which stain with osmium, and are seen in electron micrographs as random deposits lining the lumen surface (see Figure 19B and Figure 28). I do not think these chemicals help make up the wax extractives layer. I believe zone 3 (Figure 28) is a wax extractives zone and is composed of waxes such as linoceric acid, linoceryl alcohol, hydroxypalmitic acid, and 11 hydroxylauric acid and other unsaturated hydroxy acids (24). Even though most of these waxes contain functional groups, I believe they do not stain because their functional groups are not free but are involved in ester or ether linkages. Kurth (24) and Fang (11) have shown waxes to be esterified to phenolics and Kurth has shown the waxes to be linked together by

Figure 28. Untreated cork sections showing deposits of tannins, dihydroquercetin and other extractives



ether bonds.

I believe zone 2 (Figure 21 C), the suberin layer to be composed of alternating lamellae of phenolics and waxes. The phenolic material causes the phenomena of autofluorescence in the cork while the waxes cause birefringence. The phenolics are most likely composed of cinnamic acid derivatives which are the more oxidized acid precursors to coniferyl alcohol, the lignin monomer. The waxes are composed of compounds comparable to those found in the wax extractives layer. The wax molecules are oriented perpendicular to the cell surface.

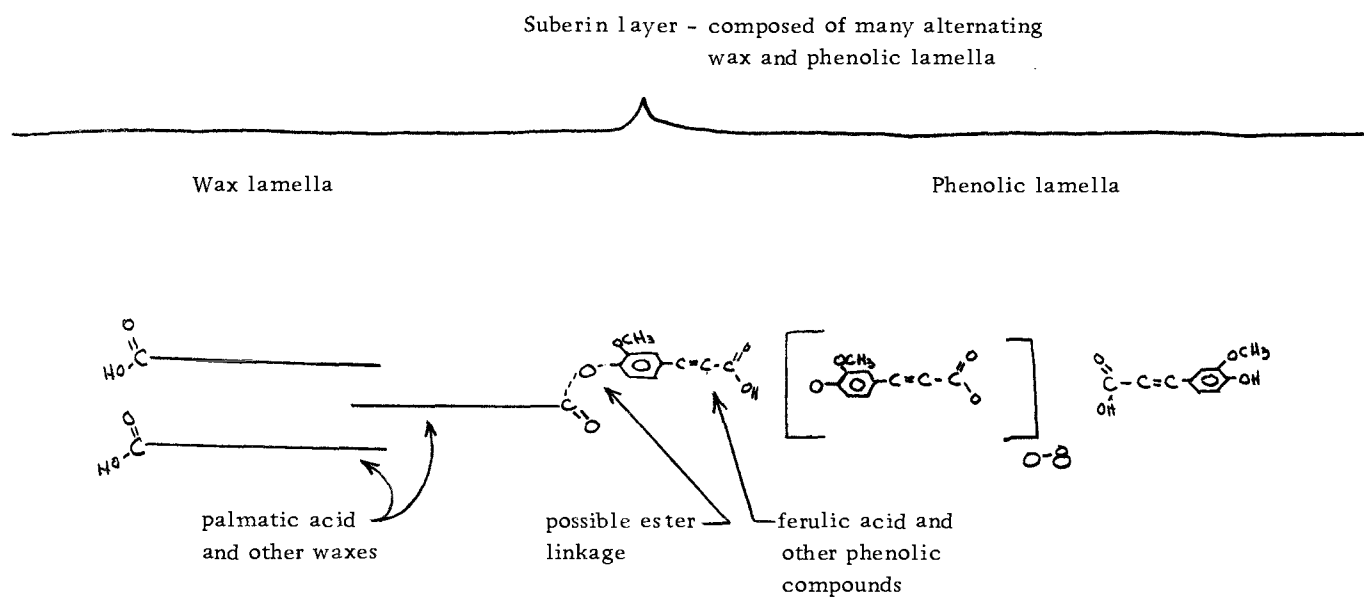
The phenolics, like lignin, are condensed to each other while the waxes have some ether linkages. At the interface where the phenolics and waxes meet, they are esterified to each other.

In transmission electron micrographs, the phenolic material appears dark and waxes light. This again is due to the fact that the phenolics have functional groups available to stain while the waxes do not. This is substantiated by the fact that most of the area in the suberin layer is dark and that 87% of the suberin layer was acetone soluble and phenolic (Table 2). Also, if we assume the wax molecules to have an average molecular weight comparable to palmitic acid 270 and the phenolic 194 comparable to ferulic acid; the weight ratio of 1.4:1 would give a molecular ratio of 1:1. Since we have a weight ratio of 12:88 or 1:7 we have a molecular ratio of one wax molecule to 5 phenolic molecules.

The thinnest wax lamella in the suberin layer has a width of 30-40 Å. If we place two hydroxypalmitic acid molecules tail to tail the width of this wax film would be approximately 30 Å and would allow for esterification to the phenolic molecule. Depending upon how the remaining 4 phenolics are condensed, the width of the phenolic layer would be 2 to 10 ferulic acid residues wide (see Figure 29). Sterically it would not be possible to bond many of the four remaining phenolic residues to the side of the phenolic residue which is esterified to the wax molecule. This would be especially true on the inner surface (the lumen side of the suberin layer). Esterification of the wax molecules to the phenolics and linkages between the wax molecules are necessary because without them the suberin layer could be removed by organic solvent extraction. Since this does not happen the waxes and phenolics must be covalently bonded. In the thick wax lamellas of the suberin layer some wax molecules may not be bonded and these can be removed by organic solvent extraction. The removal of these waxes could create the voids discussed in the Specific Gravity section (p. 39).

Materials which cause birefringence and autofluorescence are present in the suberin layer because birefringence is lost with saponification, and autofluorescence is reduced and the visible color emitted altered with saponification. The wax molecules in the suberin layer cause birefringence in the cork cell because birefringence is lost

Figure 29. Possible orientation of molecules in a wax lamella in the suberin layer using palmitic acid and ferulic acid as model compounds



when the cells are heated in glycerol (39). The wax molecules must be oriented perpendicular to the cell surface because of the results from the polarizing microscope. This brings up a very important question. Because the primary wall is composed of cellulosic microfibrils randomly oriented but parallel to the cell surface, what material is interfaced between the cellulose and the wax molecules which allows the wax molecules to be very orderly arranged perpendicular to the cell surface? This study does not answer this question but it is one which further studies should elucidate.

With regard to autofluorescence in the suberin layer, only the phenolics possess a rigid cyclic structure and electrons that could fluoresce, also paper chromatography results showed that the material which fluoresced was phenolic. Therefore the material which produces the autofluorescence observed in the suberin layer is phenolic.

One last question concerning the suberin layer needs to be discussed and this is with regard to the loss of nitrogen or protein. Originally the unextracted cork cell had 0.8 grams of protein per 100 grams o. d. cork (approximately 0.8%). After organic and aqueous solvent extraction the total protein content was lowered but the percent protein increased. Before the aqueous sodium hydroxide saponification reaction, the cork wall contained 2.8% protein; after saponification the amount of protein remaining in the residue (primary wall, middle lamella) was reduced to 2.0%. If one assumes that all the

protein before saponification was present in the primary wall" area, the protein content of the primary wall would then be 12.0%. According to Talmadge et al. (41) the normal protein levels for primary walls are 2-10%. Thus, the assumption that all the protein after organic solvent extraction is located in the primary wall of the phellem cell seems to be invalid, and indicates that some protein must be present on the primary wall or in the suberin layer. Until this question is more fully understood, protein will not be shown in my proposed model of the phellem cell (Figure 25).

The primary wall of the phellem cell is composed mostly of cellulosic material and is approximately .05 to .1 μm in thickness, which is slightly less than the figure of .1 μm used for most primary walls (22). The fact that a primary wall exists comes from studying the junction where more than two cells meet such as in Figure 18D or in Figure 21D. The analysis of replicas of cork after saponification shows that the primary wall is cellulosic. The primary wall area of such replicas shows the existence of cellulosic microfibrils (Figure 18D). This is also verified by the light microscope study which showed that only after saponification did a blue color appear indicating cellulose. Pectins and some phenolics also seem to be present in the primary wall. These data come from analysis of the transmission electron microscopy micrographs which show less intense staining of the primary wall after extraction of the pectins and by the fact that fluorescence is still exhibited by the compound

middle lamella after saponification.

The second objective of this study was to determine the nature of pitting in the phellem cell wall. Results show that the pitting is simple and all pits are eventually plugged. A significant finding was that the pits contain a material which is not oxidized by periodate and gives a callose reaction to staining with analine blue. Another fact in favor of the plugging material being callose is that callose is found only in the bark of higher plants. Although these facts are not conclusive proof of the existence of callose, they are substantial. It appears that this callose-like material at least plugs the pits from the middle lamella through the suberin layer. It could not be ascertained if the material plugs the pits from the middle lamella through the wax layer. It also appears that a carbohydrate similar to cellulose lines the pit.

The third objective of this study was to determine what holds the phellem cells together. The middle lamella between phellem cells contains phenolics and pectins and therefore is similar to the middle lamella in xylem. Previous chemical studies (15, 16) have shown that phenolics in cork are not the same as those that make up lignin in xylem elements. From the U. V. study the color change indicates that the phenolics in the middle lamella differ from those in the suberin layer. This may be a reason for the confusion that exists when trying to analyze cork for its phenolics and why "cork

lignin" has been considered to be a heterogeneous mixture originating from two or more monomers (21).

The reason for the abnormal thickening of the compound middle lamella of the tangential wall of the first formed cell in a growth ring is not known. It does occur in all growth rings and therefore is not an artifact. It may serve the function of acting as a buttress or barrier and could give the growing cell the room to expand by collapsing or crushing the older dead cork cells. Also, it is not known what component of the middle lamella is responsible for the increased thickness of the compound middle lamella. Analysis of this area using the U. V. microscope showed that this zone after saponification still had a much thicker, brighter fluorescence than lamellae of normal thickness (see Figure 17C). This thickened lamella exhibited birefringence but lost all birefringence after being saponified. This evidence would tend to indicate that the additional thickening of the compound middle lamella could be attributed to an increase in phenolics. However, the transmission electron micrograph in Figure 24A does not confirm this in that it appears from this micrograph that the increase is due to an expanded primary wall. Additional work is needed to clarify the above discrepancy.

The last item to be discussed concerns the texture found on the lumen side of the cell surface. Replicas revealed the fact that this surface of the phellem cell is very rough. After organic solvent

extraction the surface is still textured but is not as coarse as before extraction. Deposits of extractives such as those seen in Figure 28 could cause some of the roughness seen in Figure 20A but could not cause the texture seen in Figure 20B. The cause of the texture (not associated with the callose plugs) is mainly due to slight abnormalities found at the middle lamella-primary wall interface. As seen in Figure 25A and denoted by the double arrow, the slight ripple at the middle lamella-primary wall interface results in a larger bump at the lumen surface because the ripple is overlayed with other cell wall layers (i. e. the larger bumps at the lumen surface are indicative of smaller bumps beneath this surface).

CONCLUSIONS

The cell wall structure of the Douglas-fir phellem cell is composed of four zones. The outermost zone is a cellulosic primary wall. To the inside of this lies a suberin layer which is composed of alternating lamellae of phenolics and waxes. The waxes are oriented perpendicular to the cell surface and are responsible for birefringence in the phellem cell. The phenolics in the suberin layer are responsible for autofluorescence in the suberin layer of the cell wall. To the inside of the suberin layer lies a wax extractives layer. This layer is made up of randomly oriented wax molecules which are easily removed by organic solvent extraction, therefore indicating a lack of covalent bonding to the suberin layer. Lining the lumen surface is a fourth zone which is very reactive to osmium and is probably the old cell membrane and cytoplasm.

A middle lamella of phenolic-pectic materials hold the phellem cells together.

The simple pits of the phellem cell are plugged with a material which gives a callose reaction to analine blue.

The first formed tangential wall of the first formed uncrushed cell in a growth increment has an abnormally thick compound middle lamella.

EXPERIMENTAL

Chemistry

Collection and Preparation of Material

Thick periderm bark was collected from a Willamette Industries peeler log mill in Philomath, Oregon by hand-selecting the bark as it was conveyed away from the debarker and placing the bark in plastic sacks. At the laboratory the bark chunks were immediately band-sawed into bacon-like slabs 1/4-inch thick. These slabs were washed and stored in a freezer.

Later, thick periderms were excised by hand using a razor knife. Excised material was also stored in a freezer. When enough cork had been isolated the cork was immersed in liquid nitrogen and placed in a metal osterizer flask and osterized to yield a dust-like particle. These particles were then placed in round bottom (r. b.) flasks and freeze dried. The freeze dried cork powder (300 o. d. grams) was stored in desiccators.

Chemical Treatment of Cork

Solvent Extraction

Two-hundred forty-five grams of crude cork were placed in a 5-liter r. b. flask and wetted with a benzene-ethanol solution (1:1 v/v).

The flask was placed under a vacuum until all particles sunk.

The flask was then refluxed for 12 hours. There were five changes of solvent for a total time at reflux of 72 hours. At each change the cork was washed with 500 ml of ethanol. Extraction with ethanol followed for a total of 48 hours, with four changes of solvent.

After extraction with ethanol the residue cork material was air-dried and placed in tared bottles in a desiccator.

The extracts from the benzene-ethanol and ethanol series were concentrated on rotary evaporators until dry, freeze dried, and stored in a freezer for later analysis.

Hot Water Extraction

An aliquot from the benzene-ethanol, ethanol extracted cork was placed in a 500 ml r. b. flask and 400 ml of distilled water added. The cork was wetted and a vacuum pulled to sink the cork.

The cork was then filtered off and placed in a 5-liter r. b. flask containing 4 liters of distilled water and refluxed for a total of 36 hours with 3 changes of solvent.

The residue cork was freeze-dried and stored in a desiccator. All water extracts were combined and concentrated to a light syrup on a rotary evaporator. The syrup was then freeze-dried and stored in a freezer for later analysis.

Ammonium Oxalate Extraction

An aliquot from the hot water extracted cork was placed in a 500 ml flask and wetted by vacuum with a .5% solution of ammonium oxalate. The cork was filtered off and placed in a 1000 ml r. b. flask with 800 ml of .5% ammonium oxalate solution. The cork was extracted for a total of 48 hours with 2 solvent changes. At each change material was washed with distilled water. After extraction the residue was washed with 1000 ml of distilled water, and dialysed for one week.

All washes and water extracts were collected and concentrated on a rotary evaporator, freeze dried, and stored below 0° C.

After dialysis, the cork residue was freeze dried and stored in a desiccator.

Sulfite Treatment

An aliquot from the benzene-ethanol/ethanol extracted cork was placed in a 1000 ml r. b. flask, 600 ml of sodium sulfite solution (4.2 grams/100 ml water) was added, and the cork wetted by pulling a vacuum. This solution was then refluxed. The cork was refluxed for a total of 6 hours (2 hours per change, 3 changes of solvent). At each change the residue was washed with distilled water. After extraction the residue was washed with distilled water, dialysed one week, freeze dried, and stored in a desiccator. All washings were

combined, the extracts being concentrated on a rotary evaporator, freeze dried, and stored below 0° C.

Saponification

The following procedure was used for both aqueous sodium hydroxide and ethanolic potassium hydroxide saponification. A sample from the appropriate material source was obtained and placed into 4000 ml of boiling alkali and refluxed for 2 hours. There were 4 changes allowing for a total of eight hours at reflux. After treatment the residual cork was washed with distilled water and dialysed for one week. The residue was then freeze-dried and stored in a desiccator.

The spent liquors from each of the changes were pooled, neutralized to pH 6.5 with hydrochloric acid, and allowed to stand to await further analysis. The spent liquors from the sodium treatment were kept separate from those of the potassium treatment.

Acid Sodium Chlorite Treatment

1.675 grams of o. d. of sodium hydroxide saponified cork was put into a 500 ml flask with 204 grams distilled water, 30 grams sodium chlorite and 180 drops of glacial acetic acid. This mixture was put into an oven equilibrated at 85° C for 2 hours. The flask was then removed and left at room temperature for 4 hours.

The mixture was then filtered, washed and the residue ("holo-cellulose") dialysed for one week. After dialysis the residue was freeze-dried and stored in a desiccator.

Analysis of the Water Solubles from the Benzene-Ethanol Extractives

The analysis of the water solubles from the benzene-ethanol extractives followed that shown in Figure 30.

Analysis of Saponification Solubles

The analysis of the solubles from the aqueous sodium hydroxide saponification reaction followed that shown in Figure 31.

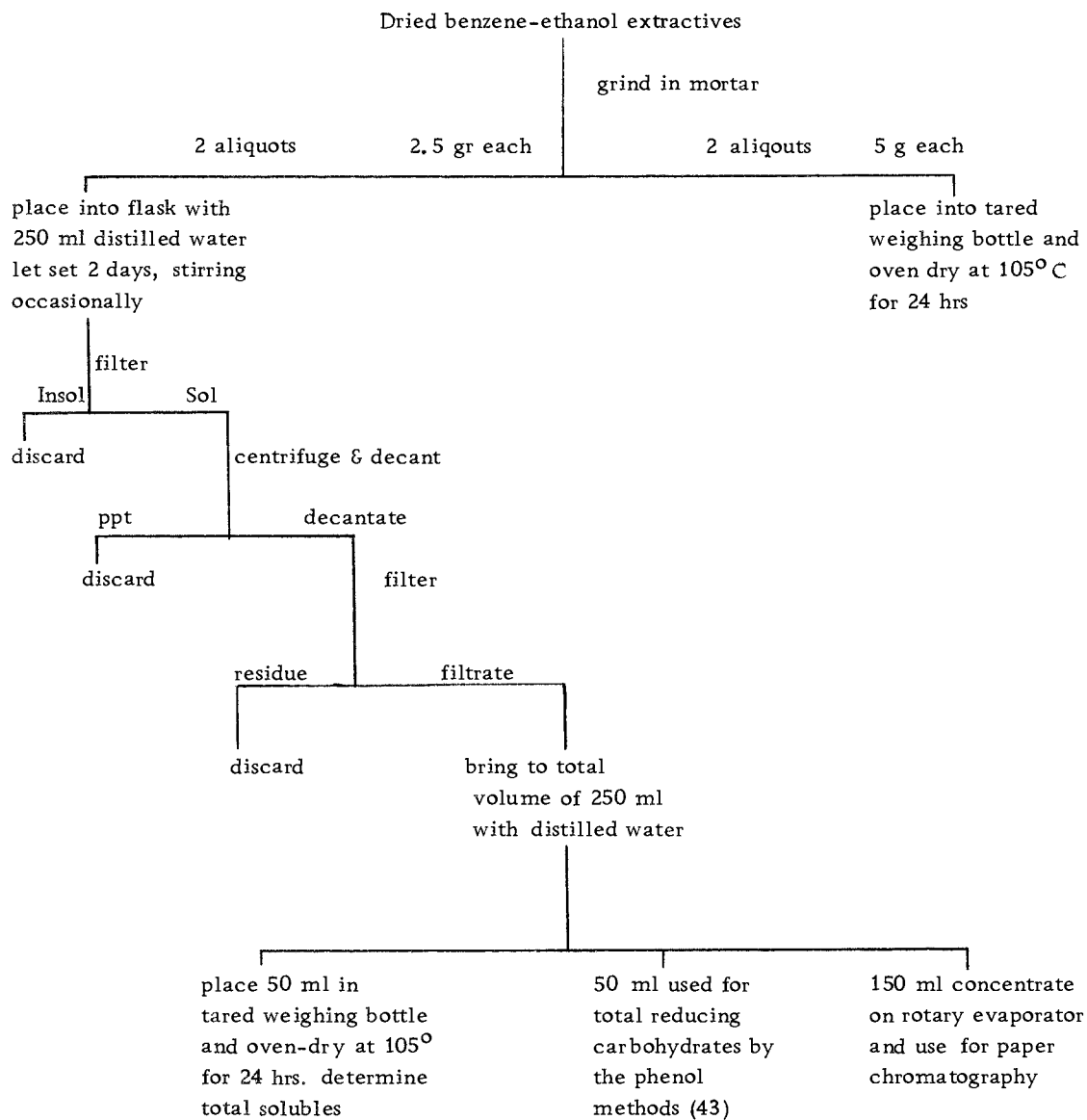
The analysis of the solubles from the ethanolic potassium hydroxide saponification reaction followed that shown in Figure 32.

Crude Protein Determination - Micro Kjeldahl Method

Principle

Samples are digested with a sulfate catalyst plus sulfuric acid and converted to ammonium sulfate. The ammonia is liberated by the addition of alkali, distilled into a boric acid solution, and titrated directly with standard acid.

Figure 30. Procedure used in the analysis of the water soluble fraction of the benzene-ethanol extractives

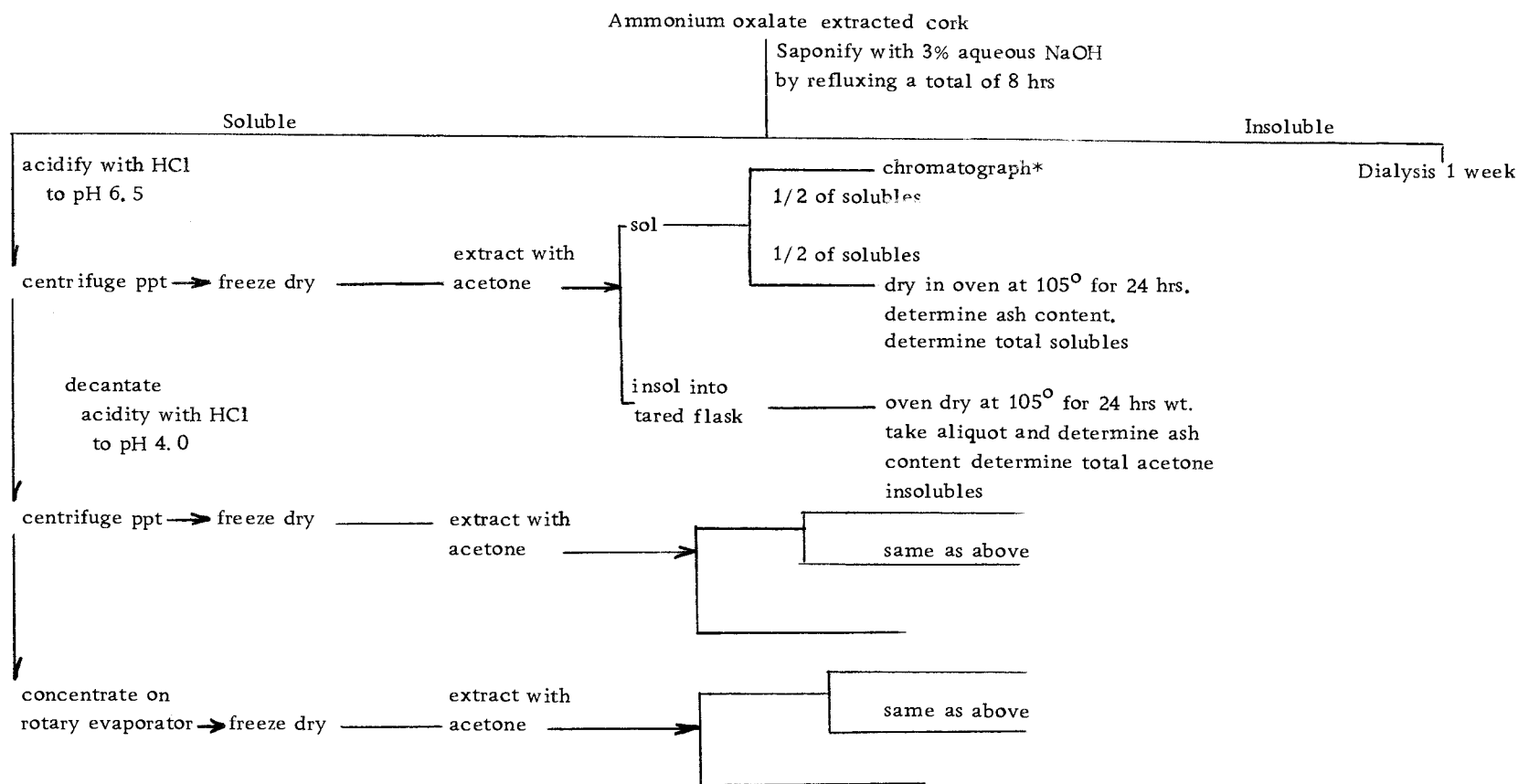


Whatmann #1 paper

solvent system of ethylacetate, pyridine, water (8:2:1, v/v)

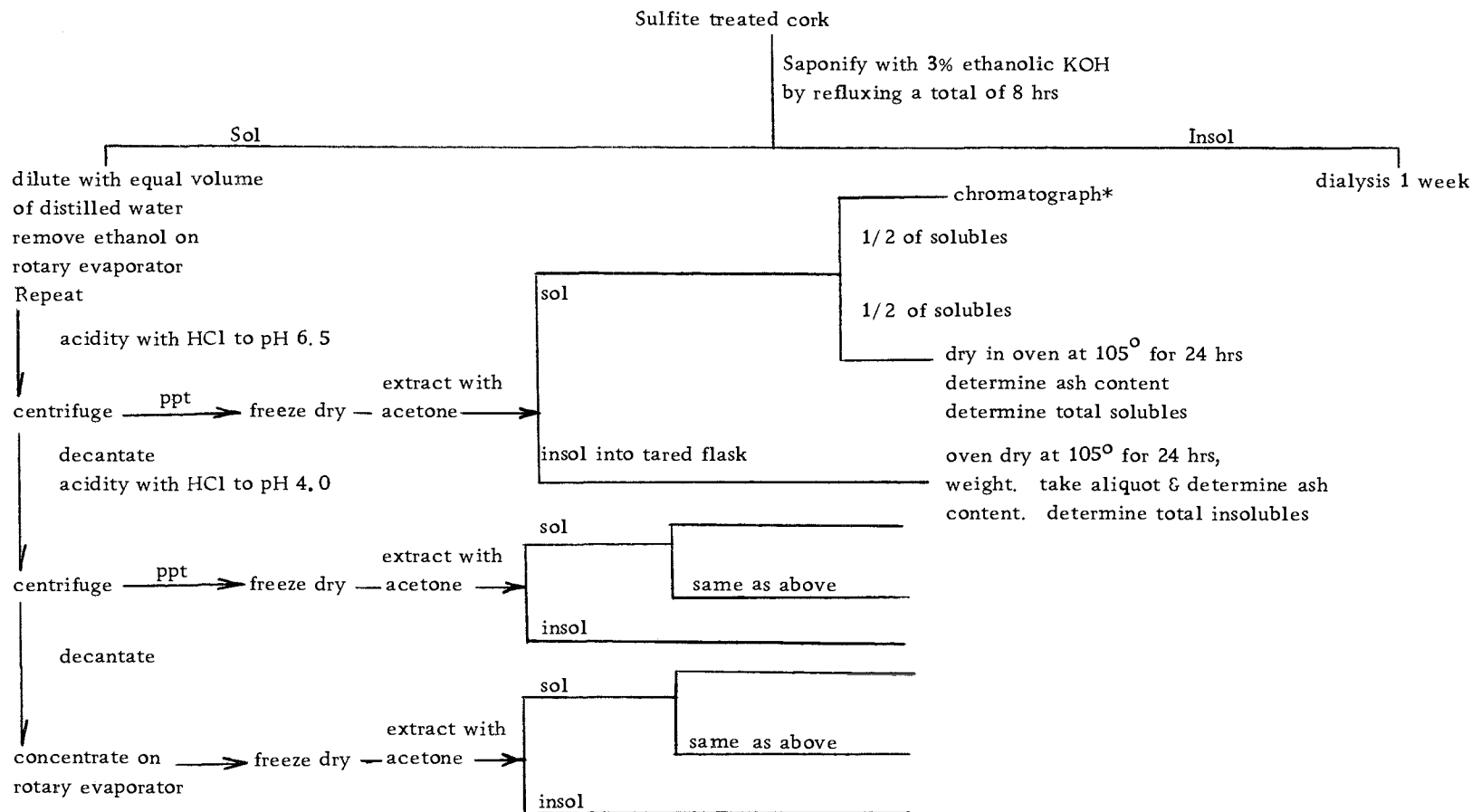
indicator spray of analine hydrogen phthalate (25)

Figure 31. Procedure used in the analysis of the aqueous sodium hydroxide saponification reaction solubles.



*paper chromatography done on whatmann #1 paper
solvent system: 2 dimensional B. A. W. and 2% A. A. (20)
1 dimensional 85 series (20)
indicator spray - ferric chloride (20) & u. v.

Figure 32. Procedure used in the analysis of the ethanolic potassium hydroxide saponification reaction solubles



*paper chromatography done on Whatmann #1 paper

solvent systems: 2 dimensional B. A. W. and 2% A. A. (20)

1 dimensional 85 series (20)

indicator spray - ferric chloride (20) & u. v.

Procedure

- a. Weighed out 0.1 gram of o. d. sample material on 1" square of weighing paper, fold, and drop into Kjeldahl flask.
- b. Add 1/4 tsp. of catalyst to each flask.
- c. Add 5 ml concentrated sulfuric acid to each flask and digest on low for 15 minutes, turn up to 3 for 15 minutes, to 7 for 15 minutes, and then to high until 1/2 hour past clearing, approximately 2 hours.
- d. Turn off all burners at once and let cool until almost room temperature. Place on rack and dilute with 20 ml of water.
- e. To 30 ml beakers add 5 ml boric acid plus 3 drops of indicator and place under condenser before Kjeldahl flask is hooked up. Make sure that the delivery tubes reach below the surface of the boric acid. Turn on the condenser water.
- f. Add 15 ml concentrated sodium hydroxide through adding funnel.
- g. Turn on steam and distill 5 minutes. After 2 minutes (approximately) indicator turns blue, distill 2 minutes more (approximately) and lower the beaker below the delivery tube for the last minute to rinse it out.
- h. Titrate to end point - 1 drop past clear.

Blank

Digest 1/4 tsp. catalyst, 5 ml sulfuric acid. Distill and titrate as above.

Calculation

$$\frac{(\text{ml H}_2\text{SO}_4 - \text{blank}) \times N \times 100 \times 6.25 \times 14.01}{\text{Sample weight} \times 1000} = \% \text{ CP}$$

$$K = \frac{(N \times 100 \times 6.25 \times 14.01)}{1000} = \text{Constant}$$

$$\% \text{ CP} = \frac{(\text{ml H}_2\text{SO}_4 - \text{blank}) (K)}{\text{sample weight}}$$

Total Ash

Bring porcelain crucibles to constant weight in muffle furnace at 550°C. Cool in desiccator and weigh. Weigh 2 grams oven dried sample into crucible, put into cool muffle furnace and heat to 550°C for 7-8 hours until contents are white. Cool in desiccator and re-weigh.

$$\frac{\text{wt of ash residue}}{\text{wt of oven dried sample}} \times 100 = \% \text{ ash}$$

Basic Method for Detection of Trace
Minerals by Atomic Absorption

1. Weight out 1 gram o. d. sample material into small

porcelain crucibles.

2. Dry in muffle furnace at 100°C 1 hour. Heat to 300°C for 1 hour and then to 500°C for 6 hours (overnight).
3. Cool samples. They can wait indefinitely at this point.
4. Dissolve in 5 ml 5N hydrochloric acid. Filter through double acid washed medium fast filter paper into 100 ml volumetric flask.
5. Rinse crucible several times with .1N hydrochloric acid.
6. Allow filter paper to drain between rinses, let it drain 1 hour to overnight with the last rinse.
7. Rinse funnels with .1N hydrochloric acid. After removing filter paper.
8. Fill to 100 ml with .1N hydrochloric acid. Invert to mix well. Analysis of Zn, Cu and Mn are now done.
9. Take a 2 ml aliquot from above (original sample) and put into 100 ml volumetrics. Put 2 ml into a 50 ml volumetric for phosphorus analysis.
10. Put in 3 ml SrCl_2 or LaCl and fill with .1N hydrochloric acid. Invert to mix well.
11. Analyze for K, Ca and Mg.

Specific Gravity Determination by Liquid Displacement

Dry samples in oven at 85°C for moisture content determination,

dry at 105° C for 24 hours, place approximately 1 gram dried material into tared volume flask (W_1). Fill flask completely with appropriate solvent, wait 15 minutes and remove any floating cork. Lower solvent, wait 15 minutes and remove any floating cork. Lower solvent level until flask is half full. Place in vacuum oven and pull vacuum; release vacuum and fill to marked line. Let sit overnight and then weight (W_2).

Let volume flask dry overnight in oven at 105° C, record weight of flask and oven-dried cork (W_3).

Rinse flask clean and fill to mark line with solvent and weight again (W_4).

Calculation of cork wall density (ρ cork) is

$$\rho \text{ cork} = \frac{W_3 - W_1}{(W_4 - W_1) - (W_2 - (W_3 - W_1))} \times \rho_s$$

$$\text{Density of displacement medium } (\rho_s) = \frac{W_4 - W_1}{50}$$

Strong Acid Hydrolysis

An aliquot of sample material was dissolved in 77% sulfuric acid and allowed to stand for 30 minutes at room temperature. Water was slowly added while stirring to provide a 3.7% sulfuric acid solution. The solution was refluxed 5 hours, cooled to room temperature,

neutralized to pH 5.0 by titration with a saturated solution of aqueous barium hydroxide. The resulting ppt was removed by centrifugation and washed well with water. The decantant plus washing were concentrated on a rotary evaporator to 50 ml.

These hydrolyzates were then subjected to paper chromatography using Whatman #1 paper. A solvent system of ethyl acetate-pyridine-water (8:2:1 v/v/v) was used with an indicator spray of aniline hydrogen phthalate (25).

The type of sample material used, their weight and the weight of acid and water added can be found in Table 5.

Table 5.- Acid hydrolysis calculations

Sample	Sample wt.	Grams of 77% H_2SO_4 acid added	Grams of water added to bring 3.0%
Raw cork	1.09313	12	224.92
Amm. oxalate treated	0.32657	9	168.69
Holocellulose	0.10881	5.2	97.47

Anatomy

Collection of Sample Material Used for Sections

The raw material source used to cut sections was the same as that used in the chemical analysis (see Chemistry section). A last

formed very thick (1/4 inch) periderm was selected. All sections used in the study were cut from this periderm using a sliding microtome. All sections except those used for replication were 24 μm thick; those for replication were 80 μm thick. After being cut the sections were placed in distilled water and stored frozen.

Chemical Treatment

Chemical treatment of the sections paralleled that of the ground cork used for chemical analysis (Figure 7). After each chemical treatment a number of sections were removed, solvent exchanged to water (or washed) and stored in distilled water.

Polarizing Microscopy

A section from each of the chemical treatments (Roman numerals I through VII from Figure 7) was mounted in glycerine on a glass slide and observed under a Zeiss polarizing microscope. Results were recorded on 35 mm Kodak Ektachrome film.

Ultra-Violet Microscopy

Two sets of sections mounted in glycerine were prepared. One set of sections was unstained and represented the series of chemical treatments found in Figure 7.

The second set of sections differed only in the fact that they

were stained with analine blue (1% aqueous solution, staining time 30 minutes) (8).

Sections were observed under bright field and ultra-violet. Results were recorded on 35 mm Kodak ektachrome film using ultra-violet light. Film development time was "pushed" by developing the film as if it had an ASA speed of 400.

Scanning Electron Microscopy

Sections representing all chemical treatments were taken and solvent exchanged into ethanol and then into trifluoromethane.

The sections were then critical point dried and mounted on scanning electron microscopy stubs using silver paint. Sections were coated with a 60/40 gold/paladium metal coating. Some sections from the 8 hour aqueous sodium hydroxide treatment were also solvent exchanged from water to acetone. These sections were then placed on scanning electron microscope stubs and allowed to air dry. After drying the sections were tacked down with silver paint and metal coated.

All sections were observed in an I. S. I. mini-scanning electron microscope model MSM-2. Results were recorded on Polaroid P/N 55 film.

Transmission Electron Microscopy

Stains

Five different staining methods were used on each of the seven different types of chemically treated cork sections.

Stain Method One - Take sections stored in distilled water and place them in a 1% aqueous solution of osmium tetroxide for 24 hours, remove and wash in distilled water.

Stain Method Two - Place sections into distilled water, adjusted to pH 2.0 with hydrochloric acid, for 2 hours. Remove sections and stain them with a 1% aqueous solution of osmium tetroxide for 2 hours, remove the sections and wash in distilled water.

Stain Method Three - Place sections into a 1% aqueous solution of sodium hydroxide for 20 minutes at room temperature. Remove sections and place into distilled water (adjusted to pH 2.0 with hydrochloric acid) for 2 minutes. Remove sections and stain with a 1% aqueous solution of osmium tetroxide for 2 hours, remove the sections and wash with distilled water.

Stain Method Four - A cellulose stain as described by Seligman (36) was used. It employed a periodate oxidation followed by a reaction with thiosemicarbazide to create unsaturated reaction sites.

Staining is accomplished by immersing sections treated in this way into a 1% aqueous solution of osmium tetroxide for 2 hours at 60°C.

Stain Method Five - A pectin stain described by Gee and

McCready (12, 28) was used. It employed alkaline replacement of the methyl esters of the uronic acids by hydroxylamine followed by staining with ferric chloride.

Embeddment

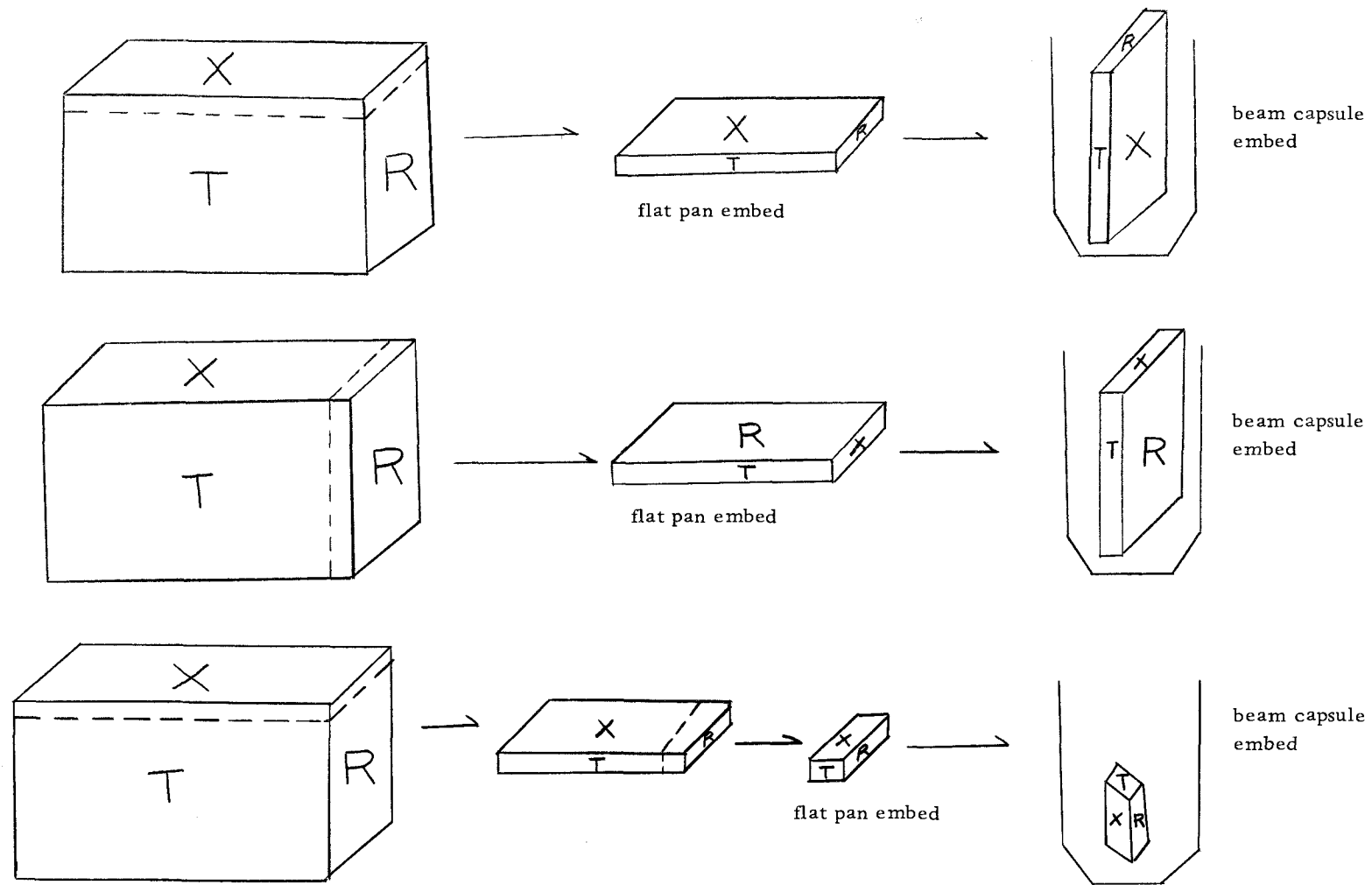
After staining sections were solvent exchanged to ethanol and then into a 50/50 mixture of ethanol and Spurr's media. Sections were flat pan embedded using Spurr's media. The ratio of components used for the Spurr's media was that described by Spurr (40): 10 grams vinyl-cyclohexene dioxide, 6 grams DES 736, 26 grams noneryl succinic anhydride and 4 grams dimethylaminoethanol. Cure time was 12 hours at 60° C.

The sections were then removed from the flat pan embeddment, trimmed and re-embedded in Beam capsules using the same Spurr's media. However, upon re-embedding into the Beam capsules, the sections were rotated 90°, thus cross sections flat pan embedded would yield radial sections for the transmission electron microscope. Likewise, radial sections yielded cross sections. Tangential sections for the transmission electron microscope were obtained from flat pan embedded cross sections (see Figure 33). In order to keep treatment types separated, a piece of paper with a number code was embedded with the sections.

Sectioning

After curing in Beam capsules (12 hours at 60° C) the embedded material was removed from the capsule and the resulting block trimmed for ultra microtomy.

Figure 33. Orientations and cutting patterns used in the embedment of cork sections for transmission electron microscopy



Sectioning of the trimmed blocks was done on a Sorvall MT-1 ultra-microtome using a diamond knife.

Silver and gold colored sections were mounted on uncoated 300 mesh copper athene grids.

Grids were examined in a Phillips Model EM-300 transmission electron microscope. Results were recorded on Kodak electron image glass plates.

Replication

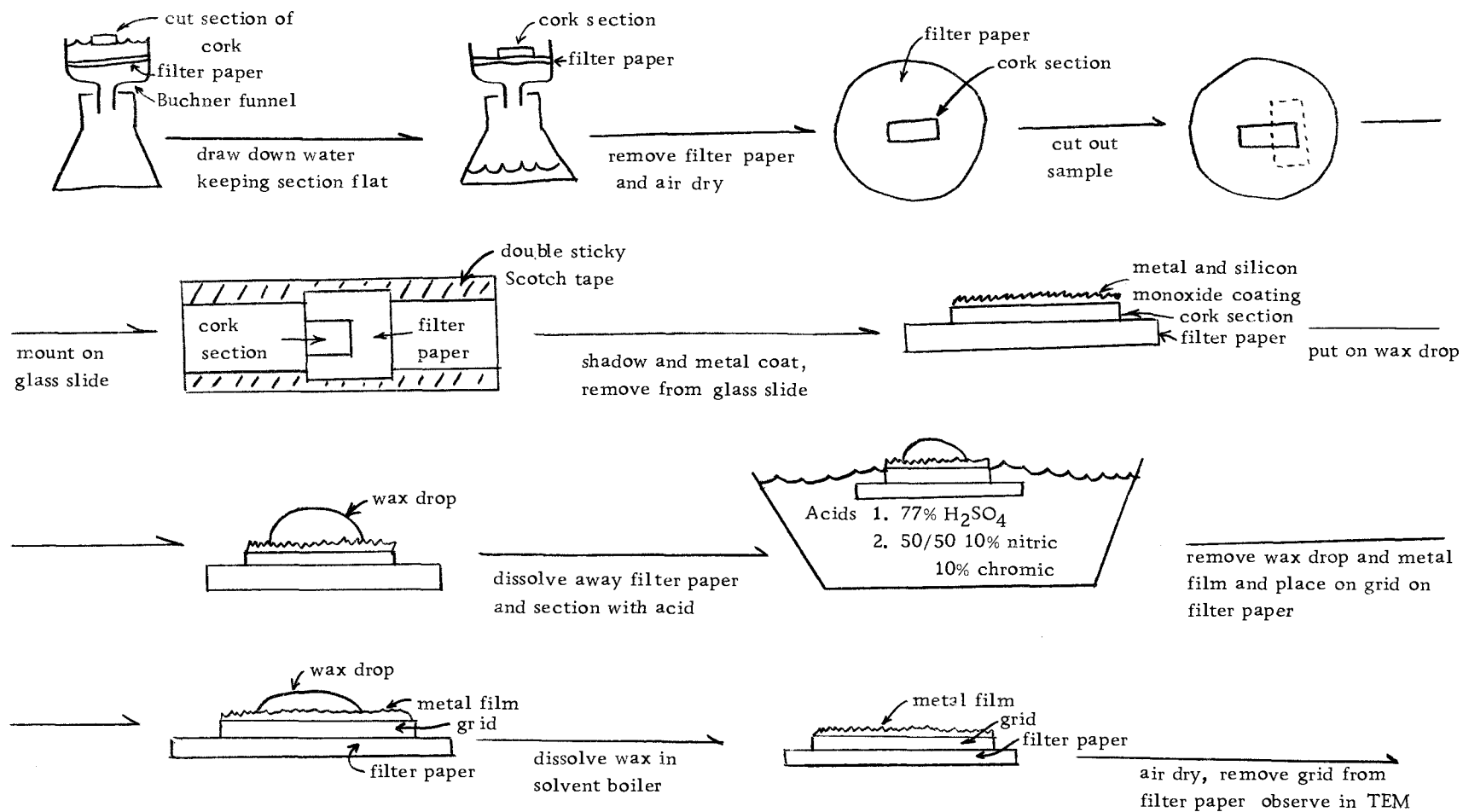
The technique used in replication was similar to that described by Côté et al. (6). Figure 34 shows the steps used in the technique.

Thick sections of cork were floated onto filter paper by draining in a Bucher funnel. The filter paper was removed and blotted on paper towels and allowed to air dry. After air drying, samples were cut out and adhered to glass slides using double sticky scotch tape.

These glass slides were put into a vacuum evaporator and the sections were shadowed with chromium and coated with a silicon monoxide film. Evaporation of the chromium was done at angles of 10, 22 and 30° while the sections remained stationary. Evaporation of the silicon monoxide film was done while the sections rotated at approximately 30 rpm.

After this a drop of wax was placed on each section. The filter paper and cork were dissolved away from the metal replica and wax with a 73% solution of sulfuric acid for 24 hours, followed by

Figure 34. Steps used in the preparation of the replicas used for transmission electron microscopy analysis



immersion in a 50/50 mixture of 10% nitric and 10% chromic acids for 24 hours.

The wax drop holding the metal replica was placed on uncoated 300 mesh copper athene grids. The wax was dissolved away by using a benzene solvent boiler. The grids holding the replica were removed and allowed to air dry.

The grids were examined in a Phillips Model EM-300 transmission electron microscope. Results were recorded on Kodak Electron Image glass plates.

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