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Doctor's Dissertation

The Methanol-Extractable Aromatic Materials in Newly Formed Aspenwood

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THE METHANOL-EXTRACTABLE AROMATIC MATERIALS IN NEWLY FORMED ASPENWOOD

A thesis submitted by

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TABLE OF CONTENTS

INTRODUCTION

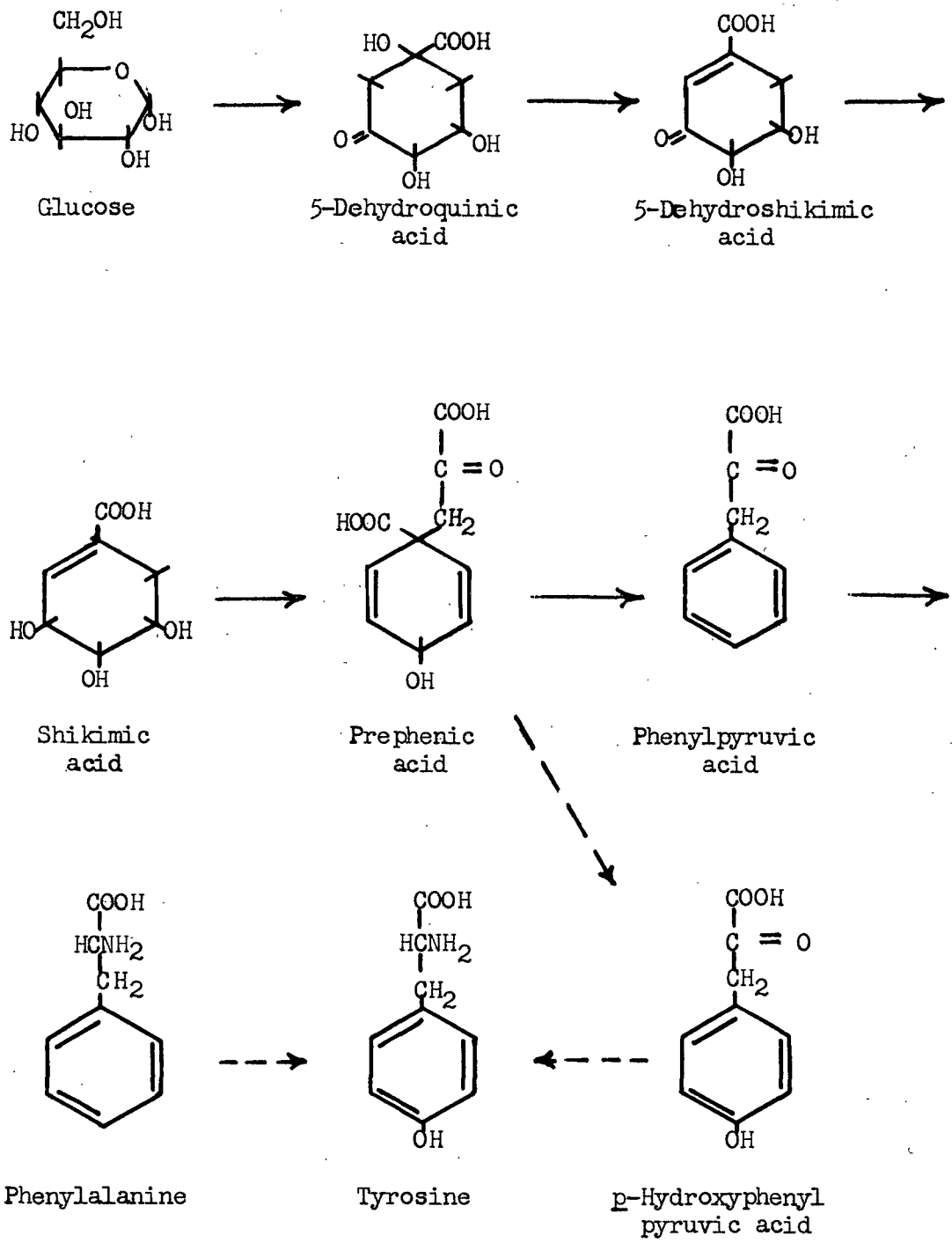
For many years wood chemists have studied lignin. However, in spite of a vast amount of research, there are still many unanswered questions about this important component of wood. One question which has long been of interest concerns the biosynthesis of lignin. For some time the general nature of the morphological changes occurring during the formation of new wood have been understood. It is known that during the growing season the cambial cells repeatedly divide and the new cells differentiate into the xylem or phloem. The newly formed cells then undergo a period of cell-wall thickening during which layers of cellulose are added internally to the original thin wall. About the time the last layers of cellulose are being added, lignin is deposited in the middle lamella and in the cell wall, cementing the fibers firmly together (1).

Until recently little experimental data were available about the substances from which lignin is formed. Apparently these metabolic intermediates are so transitory or occur in such small amounts that generally they have not been detectable by the older methods of plant analysis. Now, however, new techniques have greatly advanced the minuteness and the accuracy with which biochemical studies can be made. As a result, many of the essential chemical sequences which occur during the growth of plants have been outlined (2). Still, relatively little biochemical work has been done with lignin, and furthermore, most of that done has involved studies of model systems or simple annual plants. Thus, there is a need for further information about the biosynthesis of lignin in trees. It was with the intent of partially fulfilling this need that the present study was begun.

HISTORICAL REVIEW

The current theories of the biosynthesis of lignin were summarized and much of the evidence supporting these theories was reviewed by Adler (3). From this summary it can be seen that four stages have been postulated for the development of lignin. These are: (1) the formation of carbohydrates from carbon dioxide, (2) the formation of phenylpropane compounds from the carbohydrates, (3) the formation of the lignin monomer(s) from the first phenylpropane compounds formed, and (4) the polymerization of the lignin monomers to lignin. There is little doubt that carbohydrates are the first materials to be produced by the photosynthetic reactions (4) and therefore they must be the progenitors of all other plant materials. The next three stages of lignin development are not so well established.

The development of phenylpropane compounds from the carbohydrates is thought to proceed by the pathway shown on page 3. This sequence was established by the use of bacterial mutants (5) and fungi (6), and has yet to be shown to occur in plants. However, some evidence indicating that the pathway may be representative of plant reactions also has been reported. This evidence was obtained by injecting some of the proposed precursors, labeled with C^{14}, into young plants (7-9). Later the cell wall materials of the plants were oxidized with nitrobenzene and the C^{14} content of the aromatic aldehydes formed by the oxidation was determined. Comparing the quantity of C^{14} applied to that recovered as vanillin, syringaldehyde or p-hydroxybenzaldehyde gave the efficiency of conversion of the components to lignin. The assumption upon which this work was based was that precursors of lignin would be converted to lignin with a high efficiency.


Glucose
5-Dehydroquinic acid
5-Dehydroshikimic acid
Shikimic acid
Prephenic acid
Phenylpyruvic acid
Phenylalanine
Tyrosine
p-Hydroxyphenyl pyruvic acid

When injected into young wheat plants, shikimic acid, phenylalanine, tyrosine, and cinnamic acid were efficiently converted to lignin (7,8). Phenylpyruvic acid, which according to the theoretical pathway should have been converted with an efficiency equal to that found for phenylalanine, was actually utilized only one-fifth as well. Vanillin and p-hydroxybenzoic acid were converted to lignin with significant efficiencies, but anisic-, benzoic-, protocatechuic-, and trimethyl gallic acids were only slightly used by the plants. Sugar cane was also able to use shikimic acid in the formation of lignin (9).

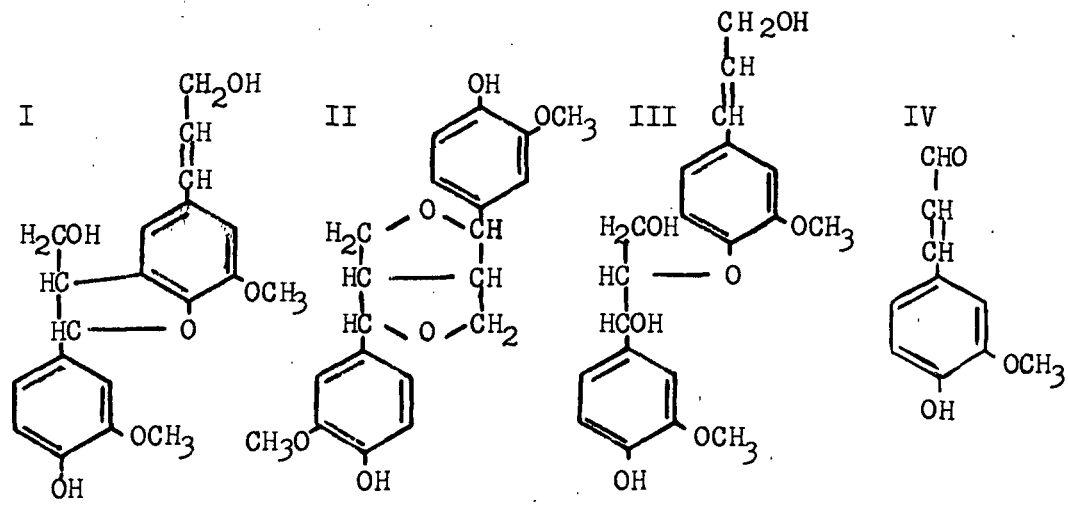
When the same type of experiments were performed with young maple, balsam poplar and caragana plants*, shikimic acid, phenylalanine and protocatechuic acid again were found to be efficiently converted to lignin, but tyrosine was not (7). The reason for this difference was not known.

Only part of the compounds included in the pathway have been found in woody plants. Shikimic acid has been reported to be widely distributed in the leaves and fruit of trees (10-12). Shikimic- and 5-dehydroshikimic acid occur in the seeds of star anise trees (Illicium species)(6) and esters of shikimic acid have been found in cambial extracts of Western hemlock (Tsuga heterophylla)(13). Phenylalanine and tyrosine are common constituents of plant protein (14), and have frequently been found free in plants (14-17).

The nature of the lignin monomer or monomers is a much disputed subject. However, it is generally agreed that these compounds have a phenylpropane nucleus (18). Freudenberg's theory of lignification, which has received

*Caragana arborescens, a hardy, shrubby tree introduced from Siberia.

more attention than any other, states that softwood lignin is formed by the polymerization of coniferyl alcohol which has been enzymatically liberated from coniferin (19). In support of this concept, Freudenberg showed that when coniferyl alcohol was treated with mushroom oxidase, a polymer (DHP) resembling Braun's spruce lignin was formed (20). By halting this polymerization at an early stage, Freudenberg isolated from the reaction mixture, four "secondary lignin building stones", dehydrodiconiferyl alcohol (I), d,l-pinoresinol (II), guaiacylglycerol-β-coniferyl ether (III), and coniferaldehyde (IV)(21,22). These same four compounds were later found in



cambial sap of spruce (23). In another study, it was found that upon ethanolysis, the coniferyl alcohol DHP yielded "Hibbert's ketones". This reaction has been judged to be one of the definitive reactions of lignin (24).

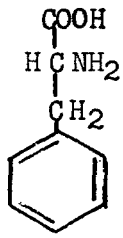
For the formation of hardwood lignin, Freudenberg suggested that sinapyl alcohol, derived from syringin, copolymerizes with coniferyl alcohol (19). A mixture of the two compounds when treated with mushroom oxidase

formed a polymer which had many of the physical properties of Braun's beech lignin (25). When sinapyl alcohol alone was treated with the enzymes, the reaction product did not resemble any lignin preparation. However, the material did yield "Hibbert's ketones" when subjected to ethanolysis (24).

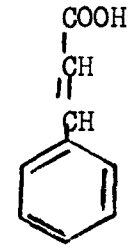
The biosynthesis of coniferyl and sinapyl alcohols from phenylalanine has been suggested to occur as follows (3): Experiments with labeled compounds as previously described indicated that ferulic acid was efficiently converted to lignin (7). Young spruce plants injected with labeled phenylalanine and ferulic acid yielded radioactive "Hibbert's ketones" upon ethanolysis. This was taken as an indication that the proposed reduction of the carboxyl groups to primary alcohol groups actually occurred (22). Serine, glycine, and methionine were shown to be capable of contributing methyl groups for the methoxylation of phenols (26, 27). When plants were injected with labeled ferulic acid and subsequently oxidized with nitrobenzene only the vanillin obtained had any radioactivity (7), indicating that syringyl groups are not formed by methoxylation of ferulic acid.

The compounds included in the proposed developmental pathway of coniferyl- and sinapyl alcohols have been found in relatively few species, as Table I shows.

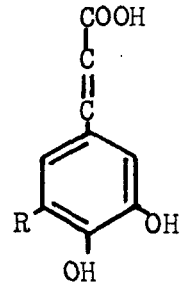
Many compounds other than those mentioned here have been postulated to be precursors of lignin (29, 30), but most of these suggestions have little experimental basis and do not mesh with the current views of lignin biosynthesis.



Phenylalanine



Cinnamic acid



Caffeic acid, R=H
3,4,5-Trihydroxy Cinnamic acid, R=OH

+

$HOCH_2CHNH_2COOH$

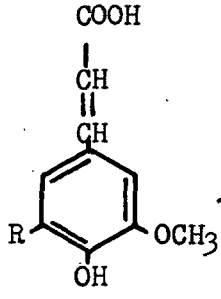
Serine

H_2CNH_2COOH

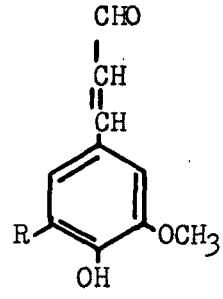
Glycine

$CH_3SCH_2CH_2CHNH_2COOH$

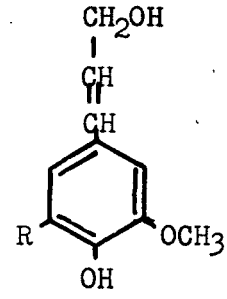
Methionine



Ferulic acid



Coniferaldehyde, R=H



Coniferyl alcohol, R=H

Sinapic acid, $R=OCH_3$ Sinapaldehyde, $R=OCH_3$ Sinapyl-alcohol, $R=OCH_3$

TABLE I

OCCURRENCE OF SOME POSSIBLE PRECURSORS OF LIGNIN (28)

Compound	In Trees (No. of species)	In Other Plants (No. of species)
Coniferin	12	2
Syringin	27	0
Coniferyl alcohol	0	1 (as benzoate)
Sinapyl alcohol	0	0
Coniferaldehyde	1	0
Sinapaldehyde	1	0
Ferulic acid	3	3
Sinapic acid	0	3
Cinnamyl alcohol	6	1
Cinnamylaldehyde	11	1
Cinnamic acid	15	3
p-Hydroxycinnamic acid	13	6
Caffeic acid	35	20

Another problem related to the biosynthesis of lignin is the question of where in trees the precursors of lignin are formed. From the results of his investigation, Wardrop concluded that they originate within the individual cells at a particular state of their differentiation (31). Contrary to this, Freudenberg feels that the lignifying tissues do not have the capacity for biosynthesis, and that, therefore, the lignin precursors are probably formed outside of the zone of lignification (32). The experimental evidence used to defend these two positions has been reviewed (31), but unfortunately most of this evidence can be used to support either side of the question. As Table I showed, some aromatic compounds have been found to occur in wood, but no thorough study of the lignifying tissues has been made to determine if Wardrop's conclusions can be defended on the basis that numerous possible precursors of lignin are present.

PRESENTATION OF THE PROBLEM

In a complex system such as a tree, countless biochemical reactions are required to form the many plant components (3). In spite of our current knowledge of biosynthetic processes, it is not possible to say for many of these reactions, what the reactants and reaction products are or even in what part of the tree the reactions occur. However, in a recent study (33) of the newly formed tissues of aspenwood, several water-soluble materials which gave positive results for two lignin color tests, i.e., the Mäule and Wiesner tests, were found. Since the tissue in which these materials were found was undergoing lignification, and because their water solubility indicated a low molecular weight, there was a possibility that the materials were precursors of lignin. Thus, the problem with which this study was concerned was: Does newly formed aspenwood* contain compounds which support of refute any of the theories about lignin precursors and their origin?

While designing the experimental program to investigate this problem, it was realized that besides answering questions concerning lignin development, information about the chemical differences between trees might also be obtained. Such information would be of value not only to plant geneticists, but also to those who are concerned with techniques of tissue sampling for studies of wood (34). Thus, the secondary problem arose: Can the differences in the composition of the methanol extractives from Populus tremuloides and Populus grandidentata trees be correlated with the species, sex, tree condition or growth site?

*Aspenwood is used here to mean Populus tremuloides only.

SUMMARY OF EXPERIMENTAL RESULTS

The solids extracted from the new xylem and other tissue taken from 13 P. tremuloides and 5 P. grandidentata trees cut during the 1956 growing season, and from 4 P. tremuloides trees cut during the 1957 growing season, were fractionated according to their solubility in neutral solvents. Examination of these materials gave the following results:

1. The Maüle- and Wiesner-positive spot which Sultze found when new xylem extracts were chromatographed and which he considered to be a single substance, was shown to be composed of at least 10 different materials.

2. Free sinapaldehyde was demonstrated to be present in aspen xylem by comparison of the chromatographic properties, color reactions, melting point, and ultraviolet spectrum of a material isolated from the xylem extracts with sinapaldehyde.

3. The amount of free sinapaldehyde present in aspenwood was found to range from 0.063% in the new xylem to 0.009% in the heartwood.

4. A Wiesner-positive substance, designated Compound K, was shown to be extracted in methanol at room temperature or higher, but not at 0°C. Although its chromatographic properties indicated that it was coniferaldehyde, its melting point and infrared spectra were greatly different from those of coniferaldehyde.

5. A substance isolated from the new xylem extracts was found to be similar to Brauns' aspen lignin in regard to its physical appearance, solubility properties, color reactions, chromatographic properties, methoxyl content, infrared spectrum, and ultraviolet spectrum in neutral and in alkaline solution.

6. A substance which had the same chromatographic properties and which gave the same color reactions as Braun's aspen lignin, but which was not precipitated in water was found in the extracts of aspen xylem.

7. The presence of phenylalanine, tyrosine, serine, glycine, and nine other free amino acids in new xylem and year-old xylem was demonstrated.

8. The relative amounts of phenylalanine and tyrosine present in new xylem was found to be larger than those in year-old xylem, i.e. 0.0024, 0.0018, and 0.0002, 0.0001%, respectively.

9. Chromatographic evidence indicated the presence of p-hydroxybenzoic acid in xylem scrapings, soft xylem, and new xylem.

10. Mäule- and Wiesner-positive materials were found in the one phloem extract examined, but these materials did not correspond chromatographically with those in the xylem extracts.

11. Chromatography of the extracts of the xylem scrapings, soft xylem, and new xylem gave no evidence of the presence of shikimic acid, ferulic acid, sinapic acid, coniferyl alcohol, coniferaldehyde, or syringin.

12. The Mäule test as used on paper chromatography was found to give a visible reaction with a spot containing as little as 10^{-7} moles of syringaldehyde. The Wiesner reagent produced a color (orange) with a spot containing 10^{-7} moles of vanillin.

13. None of the five spray reagents used on the chromatograms of the xylem extracts revealed any differences between aspen trees which could be attributed to the sex, physical condition, or growth site.

14. No differences were found between the xylem extracts of P. tremuloides and P. grandidentata.

15. Six materials, which reacted with none of the spray reagents, but which fluoresced strongly when viewed on chromatograms, were found in five of the seventeen P. tremuloides trees examined.

16. Gamma-pyrone was synthesized for comparison with materials in the xylem extracts. From this, it was possible to revise the infrared spectrum of the compounds reported in the literature.

17. 1-Fluoro, 2, 4- dinitrobenzene was found to react readily with syringaldehyde to produce a crystalline compound.

GENERAL METHODS

COLLECTION PROCEDURES

Five types of tissue were collected from P. tremuloides trees for this study. These were phloem, xylem scrapings, soft xylem, new xylem and mature wood (See Figure 1). The latter four types of tissue represent stages of lignification, namely, unlignified cells, incipient lignification, advanced lignification and completely lignified tissue. New xylem and year-old xylem were also taken from several P. grandidentata trees.

The procedures used to collect the tissue were the same as those described by Sultze (33). After a tree was felled, the branches were removed and the bole cut into billets for easier handling. Leaf specimens were brought back to The Institute of Paper Chemistry for positive identification of the tree species by members of the Wood Technology Group. A disk cut from the base of each tree was used for basal diameter and age determinations.

No collections of any tissue except mature wood were made more than three hours after a tree was felled, and all tissue was placed immediately in methanol as it was removed from the billets. This was done to halt enzyme activity.

The technique used to remove each type of tissue was as follows: Phloem. The outer bark was scraped from the billets with a potato peeler. Then the phloem was stripped off, cut into pieces and placed in methanol. Since all collections were made during the growing season the phloem was

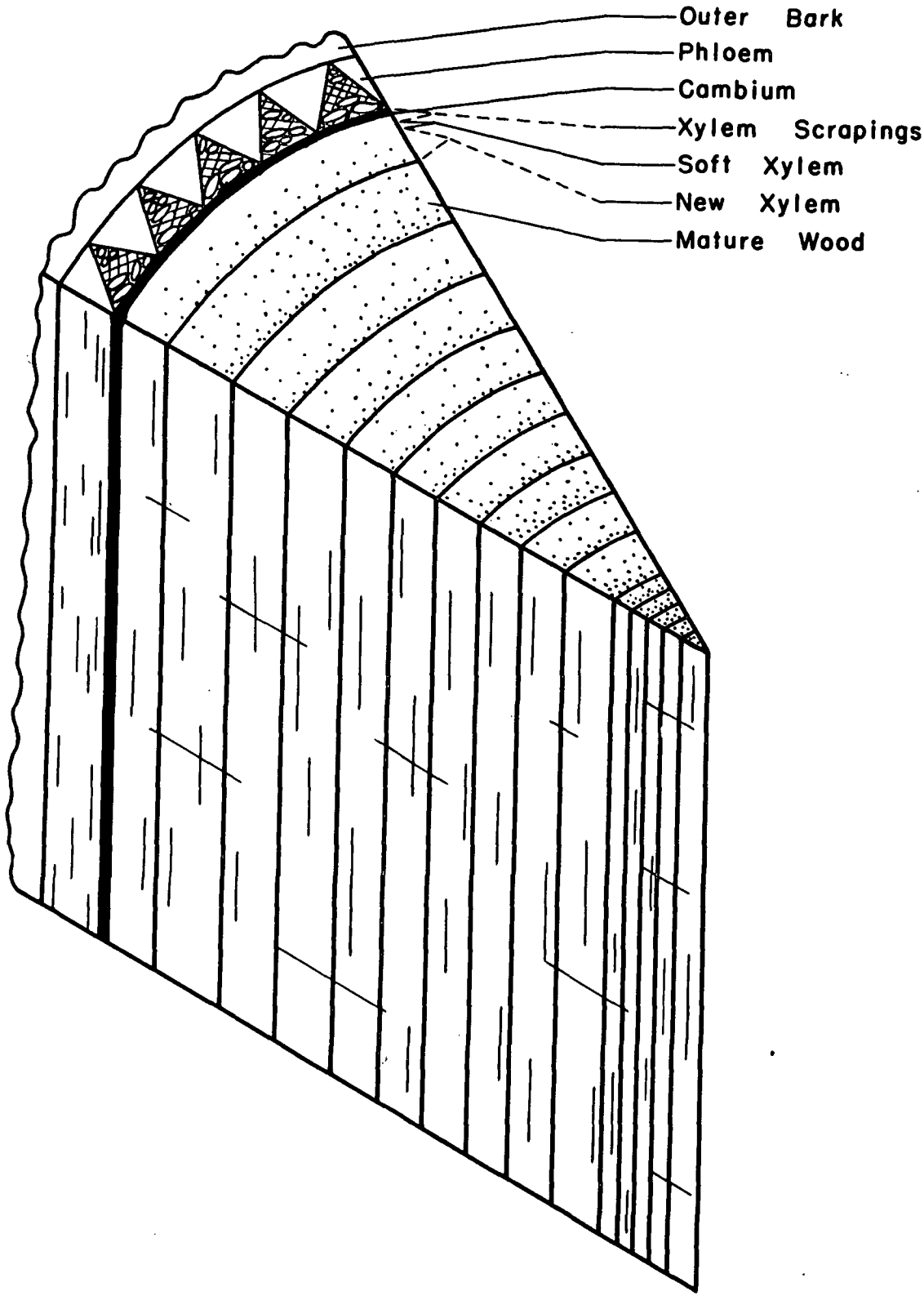


Figure 1. Cross Section of the Bole of an Aspen Tree During the Growing Season

easily peeled and thus any chance of confusing phloem with xylem was eliminated.

Xylem Scrapings. After removal of the phloem, the xylem scrapings were taken by scraping the billet lightly with a knife held perpendicular to the log surface. Only during the first part of the growing season was there a sufficient quantity of this material to warrant collection. Xylem scrapings are unlignified (33) and represent the cambium and a few layers of incompletely differentiated xylem cells.

Soft Xylem. After the xylem scrapings had been taken, the saft xylem was removed in thin, translucent ribbons, not unlike onion skin, by drawing a jackknife lengthwise along the face of the billet in such a way that the blade was nearly parallel to the surface. When xylem scrapings were not being separated, the xylem scrapings and soft xylem were removed together as soft xylem. The lignin content of the soft xylem averages 6.4% (33).

New Xylem. The new xylem was removed with a spokeshave after the soft xylem had been taken. The billet was shaved until the appearance of the pale yellow layer denoting the boundary between the new xylem and the year-old xylem was seen (33). New xylem has an average lignin content of 16.6% (33). It closely resembles mature wood but has a higher moisture content.

Mature Wood. Mature wood which has an average lignin content of 21%, was collected with a spokeshave in the same way in which new xylem was collected. The yellow layer between each year's growth was used as a guide when collecting the mature wood. When heartwood was collected, the younger wood was cut off with a power-driven planer.

EXTRACTION PROCEDURES

After returning the samples to the laboratory, the first extract, the methanol in which the tissue was placed at the time of collection, was decanted and two additional 1-hour extractions with refluxing methanol were made. Then all three extracts were combined and filtered to remove fiber fragments. The extracted tissue was finally dried at 105°C. for an hour and then weighed.

To prevent any possible effects due to heat, Sultze extracted newly formed aspenwood for 16 days with cold methanol (33). Therefore, before the hot extraction procedure was adopted for this study, a portion of one of Sultze's extracts was heated in refluxing methanol for 2 hours. Since paper chromatography of this heated extract revealed no change in its components, the hot extraction procedure was regarded as suitable. Paper chromatography also showed that the major portion of the extractable materials which gave positive reactions with the Mäule and/or Wiesner tests (M-W materials) were removed by the first room temperature extraction, and that a third 1-hour, hot extraction removed no additional M-W materials.

FRACTIONATION PROCEDURES

The methanol extracts were fractionated into the water-soluble materials, the hexane-soluble materials, and the methanol-soluble (water-insoluble, hexane-insoluble) materials. Then the water-soluble materials were extracted with ether in a continuous extractor for 24 hours to separate the ether-soluble materials. Figure 2 illustrates the fractionation scheme which is only a slight modification of that described by Sultze (33).

Prior to the ether extraction step, dark, gummy precipitates formed in the water solutions of five samples. These precipitates from water were separated from the solutions and dissolved in methanol.

During the ether extractions, amber, gummy precipitates formed in the ether flasks. These precipitates from ether were dissolved in methanol after the ether solutions were poured off.

CHROMATOGRAPHIC PROCEDURES

PAPER CHROMATOGRAPHY

Whatman No. 1 paper was used for all paper chromatograms unless other paper is mentioned. After their development, the chromatograms were air dried and examined under ultraviolet light in the presence of ammonia fumes. This allowed the use of reference materials which fluoresced but which did not react with the spray used on a chromatogram, e. g., p-hydroxybenzoic acid could be used as the reference compound on chromatograms sprayed with the Wiesner reagent.

The developers used for the paper chromatograms included:

(a) the upper phase of a toluene-acetic acid-water (4:1:5) mixture (TAW)(35);

(b) the upper phase of a butanol-acetic acid-water (63:10:47) mixture (BAW)(36);

(c) n-butyl ether saturated with water (NBEW)(37);

(d) benzene saturated with 100% formic acid (BF)(38);

(e) a mixture of phenol-formic acid-water (PFW)(3:0.01:1)(39);

(f) the upper phase of a mixture of butanol-water-acetic acid (4:5:1)(40);

(g) butanol-methyl ethyl ketone-water-17N ammonium hydroxide (5:3:1:1) (BKWAm)(40);

(h) the lower phase of a mixture of meta cresol-acetic acid water (50:2:48) (CAW)(42);

(i) the upper phase of a mixture of butanol-water-17N ammonium hydroxide (50:28:4)(BAm)(41).

When the TAW and NBEW developers were used, the chromatograms were conditioned for several hours in an atmosphere saturated with the lower phase of the developer mixtures.

The spray reagents used on the paper chromatograms included:

(a) Wiesner reagents, 1% phloroglucinol in 12% hydrochloric acid. Only the red-violet color which this reagent gives with coniferaldehydes and sinapaldehydes was regarded as a positive Wiesner reaction. The reagent also gives a red color with cinnamylaldehyde, a red-orange color with syringaldehyde, an orange color with vanillin and a yellow color with p-hydroxybenzaldehyde. The reagent produced a visible spot with as little as 10^{-7} moles of vanillin.

(b) Mäule test. The dry chromatogram was moistened over steam and placed in a covered beaker containing chlorine. After a 5-minute chlorination, the chromatogram was removed and sprayed with a 10% solution of sodium sulfite. A pink-red color was considered to be a positive reaction. The test is reasonably specific for compounds containing a pyrogallol nucleus (44). The test gave a visible color reaction with as little as 10^{-7} moles of syringaldehyde.

(c) Bis-diazotized benzidene reagent (BDB). Just before using, a 1% solution of sodium nitrite was added to an equal volume of 0.15% benzidene in 2% hydrochloric acid (76). Sufficient sodium carbonate was dissolved to

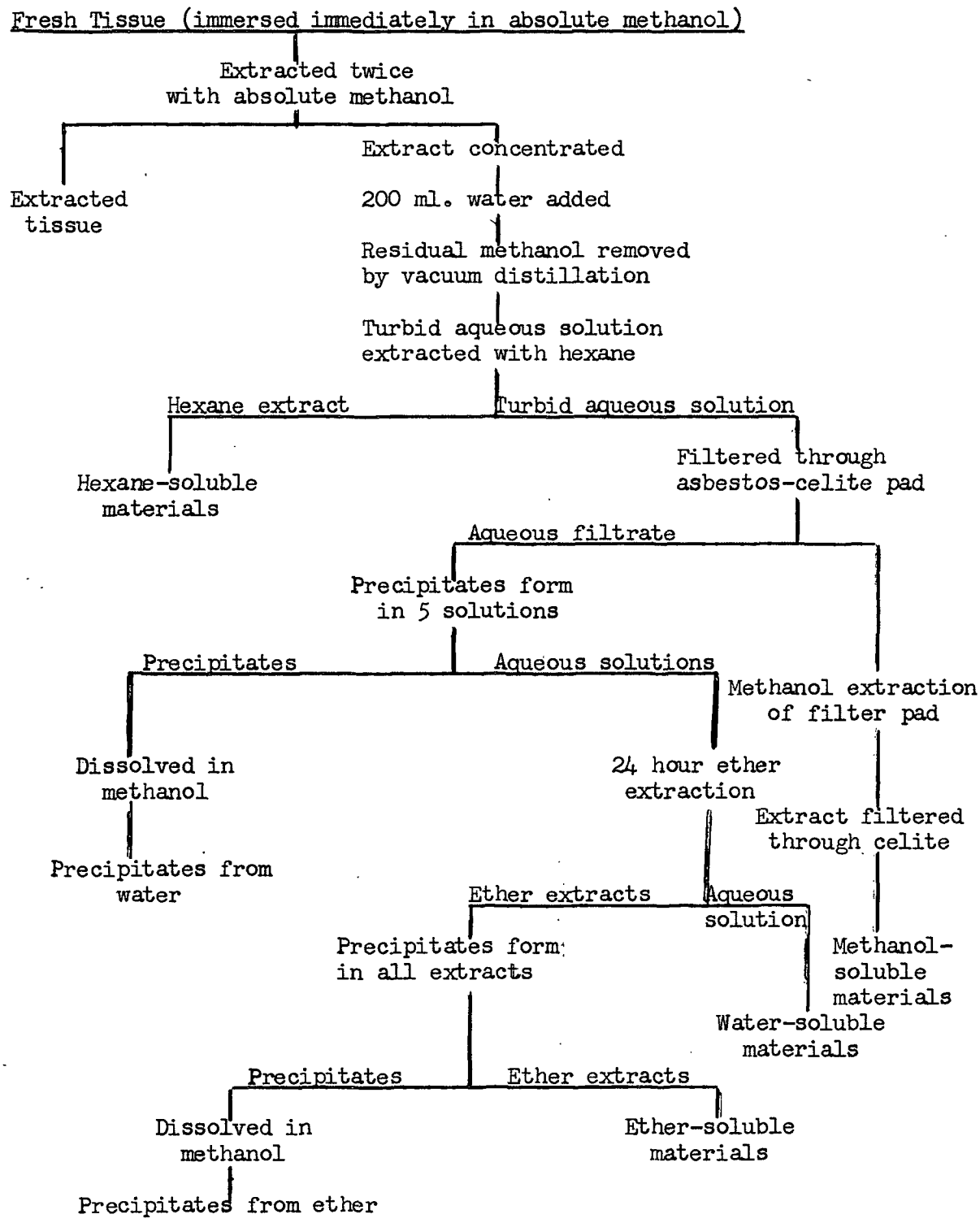


Figure 2. Flow Sheet Showing Preparation and Fractionation of Extracts

give a pale amber-colored solution. The spray reacts with phenols which are unsubstituted in either the ortho or para positions and gives a range of colors with different phenols.

(d) Ninhydrin reagent. The reagent was composed of ninhydrin in butanol containing 5% acetic acid. After a chromatogram was sprayed with the reagent, it was placed in an oven at 105°C. for several minutes in order to develop the characteristic blue color given by amino acids and amides (45).

(e) p-Anisidine reagent. Crystalline p-anisidine hydrochloride was dissolved in butanol to give a 0.5% solution (46). After spraying a chromatogram with p-anisidine reagent, the sheet was heated at 105°C. for several minutes to develop the color characteristic of aldoses.

(f) Periodate-piperazine-nitroprusside reagents. To detect hydroaromatic acids, a dried chromatogram was sprayed with a solution of sodium metaperiodate (a saturated solution diluted with two parts of water). After the sheet was dry again, it was sprayed with a solution of 0.5% piperazine and 0.5% sodium nitroprusside in 80% ethanol and heated at 105°C. for 5 minutes. A yellow-green color indicates a positive reaction (47).

(g) 2,4-Dinitrophenylhydrazine reagent (DNPH). A saturated solution of 2,4-dinitrophenylhydrazine in 12% hydrochloric acid gives a yellow-orange color with aldehydes and ketones.

(h) Propanol-hydrochloric acid. N-propanol containing 5% concentrated hydrochloric acid will give a red color with d-catechol and other materials thought to be leuco-anthocyanins (48).

COLUMN CHROMATOGRAPHY

Chromatography on columns packed with acid-washed Magnesol-Hyflo

Supercel (5:1)(49) was used to separate some of the ether-soluble materials. For applying to the columns, the ether-soluble materials were put into chloroform or benzene solution. Standard taper columns were used and after development, the packing was extruded, studied under ultraviolet light and then streaked with the desired reagent to locate bands of materials. The indicated bands were cut out and eluted by slurrying in redistilled acetone. The acetone eluate was then filtered from the Magnesol, evaporated to dryness in a rotating vacuum concentrator and the solids taken up in chloroform. The chloroform solution was used for whatever additional studies were made.

The columns were developed with petroleum ether (65-110°C.)-ethanol (50:1)(49) and benzene-ethanol (50:1).

DETERMINATION OF INFRARED SPECTRA

All determinations of infrared spectra were made with a Perkin-Elmer Model 21 Recording Infrared Spectrophotometer by Mr. Lowell Sell of the Analytical Laboratory of the Institute of Paper Chemistry. When crystalline material was to be analyzed, it was ground with potassium bromide and the mixture pressed into a transparent wafer which could be placed in the spectrophotometer. The spectra of materials not prepared as crystals were obtained from carbon tetrachloride solution of the materials. Carbon tetrachloride absorbs infrared radiation only slightly.

DETERMINATION OF ULTRAVIOLET SPECTRA

For all determinations of ultraviolet absorption, a Beckman Model DU Spectrophotometer was used. The solvent was 95% ethanol purified by refluxing with zinc powder and potassium hydroxide and then distilling from all-glass equipment (50).

SOLVENTS AND REAGENTS

All solvents and reagents used were reagent grade. In some cases, solvents were purified and redistilled. Such instances have been noted in the text.

AUTHENTIC SAMPLES OF COMPOUNDS

Authentic samples of syringaldehyde, sinapaldehyde, coniferaldehyde, p-hydroxybenzoic acid, syringic acid and ferulic acid prepared by Dr. I. A. Pearl were used for comparison with materials found on chromatograms. A sample of syringin isolated from lilac inner bark was obtained from Dr. R. E. Kremers, and a sample of shikimic acid was donated by Dr. B. D. Davis. Other compounds used were obtained from commercial sources.

COLLECTION AND PREPARATION OF MATERIAL

COLLECTION OF TISSUE

Tissue was collected during the 1956 and 1957 growing seasons. All of the trees felled grew within the Rhinelander Paper Company Experimental Forest located a few miles south of Eagle River, Wisconsin.

In 1956, new xylem was collected from thirteen P. tremuloides trees. At the same time, for comparative purposes, new xylem was taken from five P. grandidentata trees, and year-old xylem was taken from six of the P. tremuloides and from three of the P. grandidentata trees. One sample of P. tremuloides phloem was also collected.

The trees were cut at five different sites within the forest. Among the trees felled were two male and one female tree, and four diseased, injured or insect-damaged trees. The sex of the trees was determined by members of the Genetics Group of The Institute of Paper Chemistry and the trees marked accordingly. This was done by examination of the flowers which the trees bore early in the spring.

In 1957, samples of soft xylem, new xylem, and one-, two-, three-, and 18-25 year-old xylem were taken from four P. tremuloides trees. Xylem scrapings were collected from one P. tremuloides tree.

Table II summarizes the biometric data.

TABLE II

BIOMETRIC DATA

Tree	Species	Sex	Condition	Collection Period	Age, years	Butt Diameter, cm.	Height, feet
34	T	-	H	1	29	13.2	54
35	T	-	H	1	35	14.5	-
36	T	M	D	1	-	-	66
37	T	-	H	1	30	13.0	45
38	T	M	H	2	32	18.1	48
39	T	F	I	2	34	18.1	45
40	T	-	H	2	32	17.4	57
41	T	-	H	2	30	16.7	45
42	T	-	H	3	30	16.2	57
43	T	-	H	3	37	15.7	45
44	T	-	H	3	29	16.1	48
45	T	-	D	3	32	12.5	42
46	T	-	D	4	31	15.2	30
55	T	-	H	5	34	20.0	65
58	T	-	H	5	36	13.0	48
59	T	-	H	5	33	19.2	63
60	T	-	H	5	34	13.3	63
61	T	-	H	5	33	12.5	57
23	G	-	H	1	28	21.0	54

TABLE II (continued)

BIOMETRIC DATA

Tree	Species	Sex	Condition	Collection Period	Age, years	Butt Diameter, cm.	Height, feet
24	G	-	I	2	26	23.2	48
25	G	-	H	2	28	14.6	45
26	G	-	H	3	31	21.7	57
27	G	-	H	4	31	14.8	54

Species: T - Populus tremuloides
G - Populus grandidentata

Condition: H - healthy
D - diseased
I - injured

Collection Periods:
1. June 25-28, 1956
2. July 16-19, 1956
3. August 15-17, 1956
4. August 30-31, 1956
5. June 25-28, 1957

EXTRACTION OF TISSUE AND FRACTIONATION OF EXTRACTS

All tissue was extracted with methanol and fractionated by the standard procedure. Table III summarizes the fractions which were examined. Fractions not listed were discarded.

Following the fractionations, the yield of ether-soluble materials was determined. Average yield values for the other fractions have been recently reported (33). Table IV gives the average yield for each fraction. Table V gives the yield of ether-soluble materials obtained from the new xylem and the year-old xylem collected in 1956.

TABLE III

SUMMARY OF FRACTIONS PREPARED FOR STUDY

Trees	Tissue Extracted	Ether-Soluble Materials	Water-Soluble Materials	Methanol-Soluble Materials	Ppt. from Ether	Ppt. from Water	Hexane-Soluble Materials
34,35,36,37,38, 41,44,45	NX	+	+	+	+		+
39,40,42,43,46	NX	+	+	+	+	+	+
23-27(G)	NX	+	+				
38,39,40,42,44	1-X	+	+				
24-26(G)	1-X	+	+				
55	XSc	+					
58-61	SX	+					
58-61	NX	+					
58-61	1-X	+					
58-61	2-X	+					
58-61	3-X	+					
58-61	HW	+					
34	P		+ (Not ether extracted)				

NX - new xylem
SX - soft xylem
XSc - xylem scrapings
1-X - year-old xylem
2-X - two-year old xylem
3-X - three-year old xylem
HW - heartwood
P - phloem

TABLE IV

AVERAGE YIELDS OF MATERIALS EXTRACTED FROM ASPENWOOD

Tissue	Soft Xylem	New Xylem	Year-Old Xylem
Total methanol extractives, %[1]	46.1	10.2	3.3
Water-soluble materials, %[1]	41.7	8.4	1.1
Hexane-soluble materials, %[1]	1.8	0.6	0.5
Methanol-soluble materials, %[1]	2.6	1.2	1.7
Ether-soluble materials, %[2]	-	0.1	0.1

1. Calculated on basis of ovendry, ash-free, unextracted tissue (33).

2. Calculated on basis of ovendry, extracted tissue.

TABLE V

YIELDS OF ETHER-SOLUBLE MATERIALS FROM NEWLY FORMED AND MATURE ASPENWOOD

New Xylem		Year-Old Xylem	
Tree	Ether-Soluble Materials,[1] %	Tree	Ether-Soluble Materials,[1] %
34	0.05	38	0.05
35	0.06	39	0.18
36	0.07	40	0.10
37	0.05	42	0.06
38	0.05	43	0.05
39	0.17	44	0.10
40	0.08	24	0.10
41	0.06	25	0.06
42	0.04	26	0.16
43	0.09		
44	0.08		
45	0.28		
46	0.35		
23	0.07		
24	0.09		
25	0.04		
26	0.03		
27	0.04		

[1] Based on the ovendry, extracted tissue.

STUDY OF FRACTIONS

ETHER-SOLUBLE MATERIALS

PAPER CHROMATOGRAPHY OF M-W MATERIALS

At the outset, the paper chromatograms of the xylem extracts were developed with the BWA mixture recommended by Sultze (33). On these chromatograms, two Maüle-positive and one Maüle-Wiesner positive spot were found as Sultze reported. However, the latter spot was not homogeneous, i.e. parts of the spot gave different fluorescence and different reactions for the Maüle and Wiesner test; therefore, it was concluded that the spot was caused by more than one compound.

This conclusion was proven correct when the chromatograms were developed in TAW. After a 6-hour development in TAW, (the time required for the solvent front to reach the bottom of the sheet) two well-resolved spots located at $R_{vanillin}$ (R_V) 0.73 and 0.92 were found. The spot at R_V 0.73 gave a positive reaction for both the Maüle and the Wiesner tests and the spot at R_V 0.92 gave a positive Wiesner test. In addition there was a short streak running from R_V 0.02 to 0.10 which gave strong color reactions for both tests, a longer streak running from R_V 0.12 to 0.50 which gave only a positive Wiesner test and some material which did not move from the origin but which gave positive results for both tests.

Later it was found that by developing a chromatogram for 120 hours so that the faster-moving materials ran off of the sheet, the first streak (R_V 0.02-0.10) could be resolved into four spots, two of which (R_V 0.012

and 0.05) gave positive reactions for both the Mäule and the Wiesner tests and two (R_V 0.03 and 0.06) with only the Wiesner test. The second streak could not be resolved by paper chromatography, but after the ether-soluble materials were chromatographed on Magnesol columns, it was found that the streak had obscured two Wiesner-positive spots at R_V 0.20 and 0.40. The materials causing the spots and the material responsible for the streak were found in different bands on the Magnesol columns.

New xylem and year-old xylem from both P. tremuloides and P. grandidentata contained the same M-W materials although the concentration in the new xylem appeared to be several times greater than in the year-old xylem.

Paper chromatography of the ether-soluble materials isolated from the mature xylem (two- and three-year old xylem and heartwood) showed that this tissue contained the same M-W materials as new xylem. However, this was not true of the xylem scrapings and soft xylem. On paper chromatograms of the ether-soluble materials of soft xylem, the spots at R_V 0.05, 0.20 and 0.73 were found. There was also a spot at the origin, but this spot gave a orange, vanillin-type Wiesner reaction unlike the red-violet reaction given by the materials taken from new xylem and mature wood. The Mäule reaction of the spot was typical of syringyl compounds. This spot was seen on the paper chromatograms of the ether-soluble materials from the xylem scrapings also. The spot at R_V 0.73 was the only other M-W material found in the xylem scrapings extract.

The M-W materials found in the xylem extracts had different chromatographic properties from those found in the phloem extract.

The paper chromatograms of the ether-soluble materials were also sprayed with bis-diazotized benzidene (BDB) to show free phenolic groups, and 2,4-dinitrophenylhydrazine to show reactive carbonyl groups. Table VI shows the locations and reactions of each of the spots found on paper chromatograms of the ether-soluble materials when developed in TAW. Only those spots which gave either positive Maüle or Wiesner reactions are listed.

COLUMN CHROMATOGRAPHY OF THE M-W MATERIALS

Chromatography on columns of acid-washed Magnesol-Hyflo Supercel (5:1) was used to isolate some of the ether-soluble M-W materials. Paper chromatography on Whatmann 3MM paper and column chromatography on a cellulose column were also tried for this purpose but discarded when it was found that the 3MM paper contained a relatively high quantity of extractives which contaminated eluates, and that the cellulose column did not separate the materials.

Using a No. 3 column developed with 1200 ml. of the petroleum ether-ethanol developer, the material causing Spot K, Compound K, (R_V 0.92) could be isolated. To isolate other materials, it was necessary to use a two-step procedure. First, the materials to be separated were chromatographed on a No. 1 column developed with 45 ml. of benzene-ethanol. With this developer, only one Wiesner-positive band moved down the column. Paper chromatography of the eluate of this band showed that it contained the materials which caused Spots F, G, H, and K. All other M-W materials remained at the top of the column. When the eluate of the single band was

TABLE VI

PAPER CHROMATOGRAPHY OF ETHER-SOLUBLE M-W MATERIALS

Spot	Location (TAW developer)		Color Reactions					Occurrence		MW Relative Intensity of Wiesner Reaction[4]
	R_V[1]	R_{PHBA}[2]	Maule	Wiesner	BDB	DNPH	XSc	SX	NX	
A	0.0	0.0	+	+	+	+	-	-	+	+ ++++
B	0.012[3]	0.25	+	+	(not determined)		-	-	+	+ +
C	0.03[3]	0.60	-	+	(not determined)		-	-	+	+ ++
D	0.05[3]	1.00	+	+	(not determined)		-	+	+	+ +
E	0.06[3]	1.25	-	+	(not determined)		-	-	+	+ ++
F	0.20		-	+	-	+	-	+	+	+ ++
G	0.40		-	+	-	+	-	-	+	+ ++
H	0.73		+	+	-	+	+	+	+	+ +++
K	0.92		-	+	+	+	-	-	+	+ ++
L	0.12-.50 (streak)		-	+	-	-	-	-	+	+ ++
M	1.25 (In magnesol column eluates only)		-	+	-	-	-	-	+	+ +
N	0.0		+	(orange)	+	+	+	+	-	- ++

1 R_V - $R_{vanillin}$
2 R_{PHBA} - R_{para}-hydroxy benzoic acid
3 Calculated from R_V 0.05 of para-hydroxybenzoic acid
4 When spotted proportionally to the weight of tissue extracted

XSc - xylem scrapings
SX - soft xylem
NX - new xylem
MW - mature wood

chromatographed on a No. 3 column developed with 1200 ml. of petroleum ether–ethanol, Compound K and the material which caused Spot H, Compound H (R_V 0.73) were isolated. In addition to these two compounds, a third material, Substance M (R_V 1.25) was isolated. This material was found on all chromatograms of the eluates from the Magnesol columns but on none of the chromatograms of the original ether-soluble materials.

To apply the ether-soluble materials to the Magnesol columns, the ether solutions were evaporated and the solids taken up in chloroform. The chloroform solutions were poured onto the top of the packed column. Later it was found that only the materials causing Spots F, G, H, and K were benzene-soluble. Thus, by washing the dried ether-soluble materials with benzene instead of chloroform, the separation obtained on the column developed with benzene–ethanol could be more easily obtained.

IDENTIFICATION OF SINAPALDEHYDE

Paper chromatography showed that Compound H gave positive Mäule, Wiesner, and DNPH reactions and a negative reaction with BDB. Because these are properties of sinapaldehyde, Compound H was chromatographed on paper beside an authentic sample of sinapaldehyde. With the TAW developer, the two compounds were moved to R_V 0.73, and with the NBEW developer, to R_V 0.18.

To conclusively prove Compound H to be sinapaldehyde, crystals of the compound were prepared and their melting point determined as 105-6°C. (Sinapaldehyde, m.p. 109°C.) The maximum points of its ultraviolet spectrum were found to correspond to those of sinapaldehyde at 243 mmu. and 348 mmu.

To obtain crystalline sinapaldehyde, the chloroform solution of the material isolated by the Magnesol column chromatography was concentrated to a few milliliters and filtered through a tightly packed cotton plug to remove Magnesol fines. Several drops of petroleum ether were added to the solution until it became turbid. Then it was placed in the refrigerator. After some time, a few light tan crystals formed.

For the ultraviolet spectrum determination, a small amount of the ether-soluble materials was streaked across the top of a paper chromatogram which was then developed in TAW. The band of sinapaldehyde at R_V 0.73 was located by its fluorescence under ultraviolet light, cut out, and eluted with purified 95% ethanol. This eluate was used for the spectrum determination.

An attempt was made to prepare the 2,4-dinitrophenyl ether of sinapaldehyde from the solids in the mother liquor. For this, the solids were dried, weighed and redissolved in 10 ml. of acetone. To the 0.166 gram of sinapaldehyde were added 0.150 gram of 1-fluoro, 2,4-dinitrobenzene and 0.043 gram of sodium bicarbonate dissolved in 3 ml. of water. When the mixture was cooled, yellow crystals formed (m.p. 94°C). Recrystallization from acetone gave white crystals which slowly melted over the range 55-65°C. Because of the great difference in the melting points of the two preparations, no further study was made. There was not sufficient authentic sinapaldehyde available for the preparation of a derivative using pure material.

DISTRIBUTION OF SINAPALDEHYDE

The distribution of sinapaldehyde across the bole of an aspen tree was

determined by a colorimetric procedure. For this, the ether-soluble materials isolated from the soft xylem, new xylem, one-, two-, three-, and 18-25 year-old xylem of the four P. tremuloides trees cut in 1957 were used. The ether solutions of these materials were vacuum concentrated and made up to 100 ml. volumes. Aliquots of each were applied to paper chromatograms in 8-inch streaks centered on 10-inch wide sheets. The chromatograms were developed in TAW and the fluorescent bands of sinapaldehyde cut out and eluted with purified 95% ethanol. The eluates were brought to 10 ml. volumes and their optical densities at 348 mmu. determined. Duplicate determinations were made for the solutions from each type of tissue.

Using solutions of authentic sinapaldehyde, the optical density-concentration factor for this compound was determined to be 10.21 (at 348 mmu.), i.e. 10.21 times the optical density at 348 mmu. equals the concentration of sinapaldehyde in mg./l. With this factor, the average distribution of sinapaldehyde across the bole of an aspen tree was calculated. These calculations were based on the ovendry, unextracted wood weight. For this the average extractives contents of the different tissues listed in Table IV were used. The distribution of sinapaldehyde across the bole of an aspen tree is given in Table VII.

CHARACTERIZATION OF COMPOUND K

Compound K was shown by paper chromatography to give positive reactions with Wiesner, BDB, and DNPH sprays and a negative Mäule test. It was also shown that on paper chromatograms developed with TAW, the location of Compound K (R_V 0.92) corresponded with that of authentic coniferaldehyde.

On paper chromatograms developed with NBEW, spots of both compounds were found at R_V 0.46.

TABLE VII

DISTRIBUTION OF SINAPALDEHYDE ACROSS THE BOLE OF AN ASPEN TREE

Tissue	Sinapaldehyde Content, %
Soft xylem	0.026
New xylem	0.063
Year-old xylem	0.013
Two-year old xylem	0.010
Three-year old xylem	0.009
Heartwood	0.009

Crystals of what was thought to be Compound K were obtained by the same procedure by which crystalline sinapaldehyde was prepared. However, when some of the crystals were redissolved in methanol and chromatographed in TAW, a Wiesner-positive spot at R_V 0.02 was found instead of at R_V 0.92 as was expected.

When heated, the crystals decomposed at 205-8°C. leaving a black residue in the bottom of the capillary tube. A bright orange sublimate crystallized in the unheated upper portion of the tube. Paper chromatography of a methanol solution of the sublimate showed no fluorescent or Wiesner-positive spots.

Because there were not enough crystals to allow a determination of their infrared spectrum, an additional amount of Compound K was isolated by Magnesol column chromatography. This material was not crystallized but was shown to be chromatographically pure. The infrared spectrum was

determined using a carbon tetrachloride solution of the material. Figure 3 shows the spectrum obtained. From this spectrum it was apparent that Compound K contained a high proportion of aliphatic carbon linkages (band at 3.40 mu.) as compared with aromatic linkages (6.20 mu.). It is highly hydroxylated (2.85 mu.) and contains one or more carbonyl groups (6.95 mu.) possibly in ester linkages (8.0-9.0 mu.). The spectrum bore no resemblance to that of coniferaldehyde.

Because the evidence indicated that Compound K contained an aromatic aldehyde (positive Wiesner reaction) and a carbonyl group (infrared spectrum), and because Brauns' aspen lignin has been reported to contain esters of p-hydroxybenzoic acid (68), it was thought that the compound might have the same type structure as vanillin benzoate. To investigate this, vanillin benzoate (m.p. 75°C.) was prepared and its infrared spectrum determined. This spectrum was not similar to that of Compound K.

Later in the study, an experiment was made to determine if any change in the ether-soluble materials occurred during their handling or storage. For this, samples of new xylem were taken from two P. tremuloides trees, placed immediately in refrigerated methanol, and kept on ice until they could be returned to the laboratory (24 hours). Then the methanol was decanted and concentrated under vacuum at room temperature. Paper chromatography of the concentrate showed that all of the M-W materials listed in Table VI were present except Compound K. When the samples were extracted a second time with hot methanol, Compound K was found in the extract.

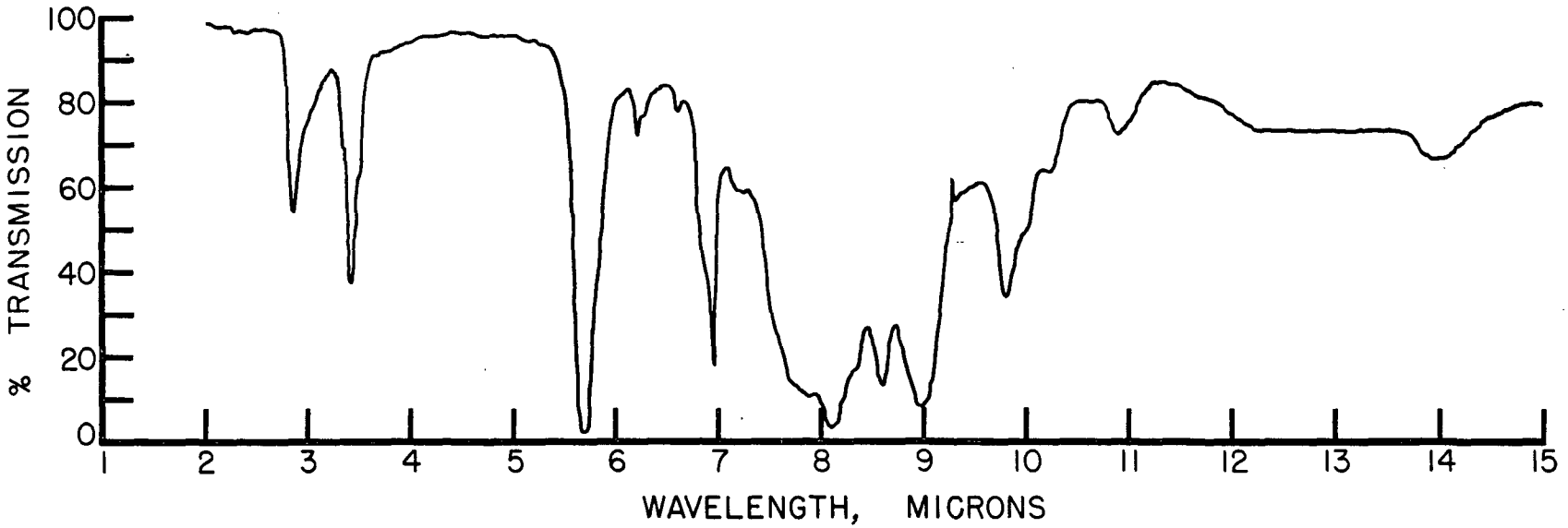


Figure 3. Infrared Spectrum of Compound K

CONIFERYL ALCOHOL

The possible presence of coniferyl alcohol in new xylem was investigated by paper chromatography of the refrigerated methanol extract used in the study of Compound K. The paper chromatogram was developed in BAm and sprayed with BDB. Coniferyl alcohol has an R_f value of 0.79 when developed in this solvent (51). The refrigerated methanol extract of new xylem gave no spot at R_f 0.79.

INVESTIGATION OF SUBSTANCE M

To obtain some idea of the nature of the artifact, Substance M, the chromatographically pure material obtained by Magnesol column chromatography was put into carbon tetrachloride solution and its infrared spectrum determined. Analysis of this spectrum (Figure 4) showed that the material contained a high ratio of aliphatic to aromatic carbon linkages (3.45 mu., 6.2 mu.), and one or more carbonyl groups including probably at least one ester linkage (6.85 mu., 7.80-9.00 mu.).

p-Hydroxybenzoic Acid

To isolate any acids in the ether-soluble materials, an ether solution of the materials from new xylem was extracted with 6% sodium bicarbonate solution. The aqueous extract was extracted with ether to remove any non-acidic entrained materials, acidified with sulfuric acid, and then reextracted with ether. This extract was spotted on a paper chromatogram and developed with BF. When sprayed with BDB, a spot at $R_{\text{vanillic acid}}$ (R_V 0.27) was found. An authentic sample of p-hydroxybenzoic acid had the same R_V.

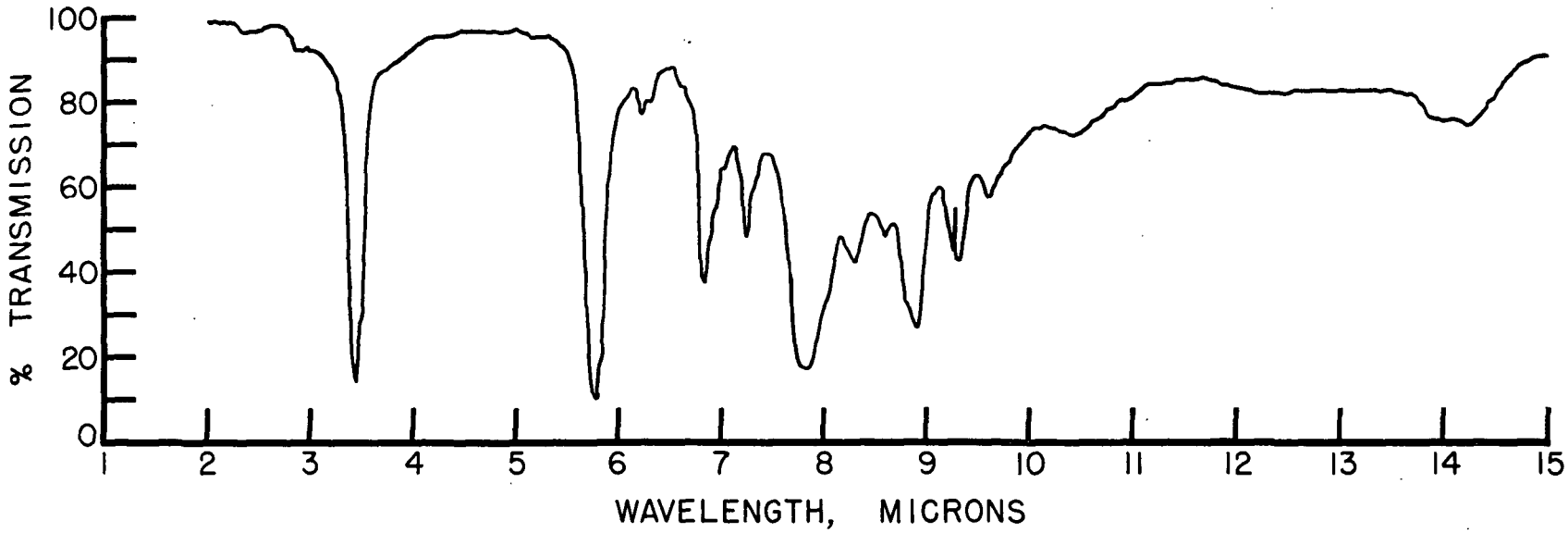


Figure 4. Infrared Spectrum of Substance M

value and the same shade of color reaction. No spots corresponding to those given by authentic samples of ferulic or sinapic acids were found on the chromatogram.

Later the procedure was used to show the presence of p-hydroxybenzoic acid in xylem scrapings and soft xylem. No other acids were found in the extracts from those types of tissue either.

HYDROAROMATIC ACIDS

To determine if the ether-soluble materials included shikimic acid or any of the other hydroaromatic acids postulated to be lignin precursors, the ether solutions from the xylem scrapings, soft xylem, and new xylem were chromatographed on paper using the PWF developer. The chromatogram was air dried for two days until no smell of phenol remained and then sprayed with the periodate-piperazine-nitroprusside reagents (12, 47). After heating the chromatogram, a yellow-green spot at R_f 0.49 was given by the soft xylem extract. A sample of authentic shikimic acid was found at R_f 0.53. None of the other hydroaromatic acids (5-dehydroquinic, 5-dehydroshikimic, prephenic acids) were available for comparison, nor was any mention of their chromatographic properties found in the literature.

WATER-SOLUBLE MATERIALS

PAPER CHROMATOGRAPHY

Paper chromatography was used to indicate the types of compounds included in the water-soluble materials. The chromatograms were developed

with BAW, then examined under ultraviolet light, sprayed with p-anisidine, ninhydrin, propanol-hydrochloric acid, and the Wiesner reagent, and given the Mäule test. From this, the water-soluble materials were shown to be a mixture of sugars (p-anisidine-positive materials) and amino acids (ninhydrin-positive materials). Several other materials were found in the fractions in which the precipitates from water formed. These materials fluoresced strongly but reacted with none of the sprays. No Wiesner-positive spots were found on the chromatograms. The water-soluble materials from new xylem did give one Mäule-positive spot.

Further investigation of the single Mäule-positive spot given by the new xylem water-soluble materials showed that it did not react with any of the other sprays and was not fluorescent. Neither did its R_f of 0.14 correspond with that of an authentic sample of syringin (R_f 0.80). No evidence of the presence of syringin could be found.

Table VIII gives the R_f values for the unidentified water-soluble materials in the solutions which yielded the precipitates from water.

TABLE VIII

CHROMATOGRAPHIC PROPERTIES OF UNIDENTIFIED WATER-SOLUBLE MATERIALS

Material	R_f (BAW developer)	Color Under Ultraviolet Light
1	0.06	white
2	0.11	white
3	0.17	light blue
4	0.31	blue-white
5	0.51	yellow
6	0.81	white

AMINO ACIDS

To make a qualitative and a quantitative analysis of the acidic and neutral amino acids in the water-soluble materials, the method of Moore and Stein (52) was used. This method involves chromatography of the amino acids on a column of ion-exchange resin developed with buffer solutions, and a colorimetric analysis of the eluate fractions after addition of ninhydrin to produce the characteristic amino acid color reaction. Identification of amino acids is based upon the fractions in which they are found.

Because of the time required to analyze a single mixture by this method, only two solutions were examined. These were the solutions of water-soluble materials from the new xylem and the year-old xylem of Tree 40. Figures 5 and 6 give the optical density of each fraction collected and the identifications made. The standard curves used for making the identifications were those prepared by Moore and Stein (52) and Dubey (53).

Table IX gives the results of the amino acid analyses. The calculations were based on the ovendry weight of the extracted wood from which the acids were taken.

To verify the presence of phenylalanine and tyrosine, two-dimensional paper chromatography was used. A mixture of phenylalanine and tyrosine was spotted on one sheet and the water-soluble materials from the new xylem of Tree 40 on another. Both sheets were then developed in BWA for 33 hours and then in BKWAm for 20 hours. The water-soluble materials gave a ninhydrin-positive spot at 23-29 (23 cm. in the direction of the first developer and 29 cm. in the direction of the second), and at 13-15. Phenylalanine

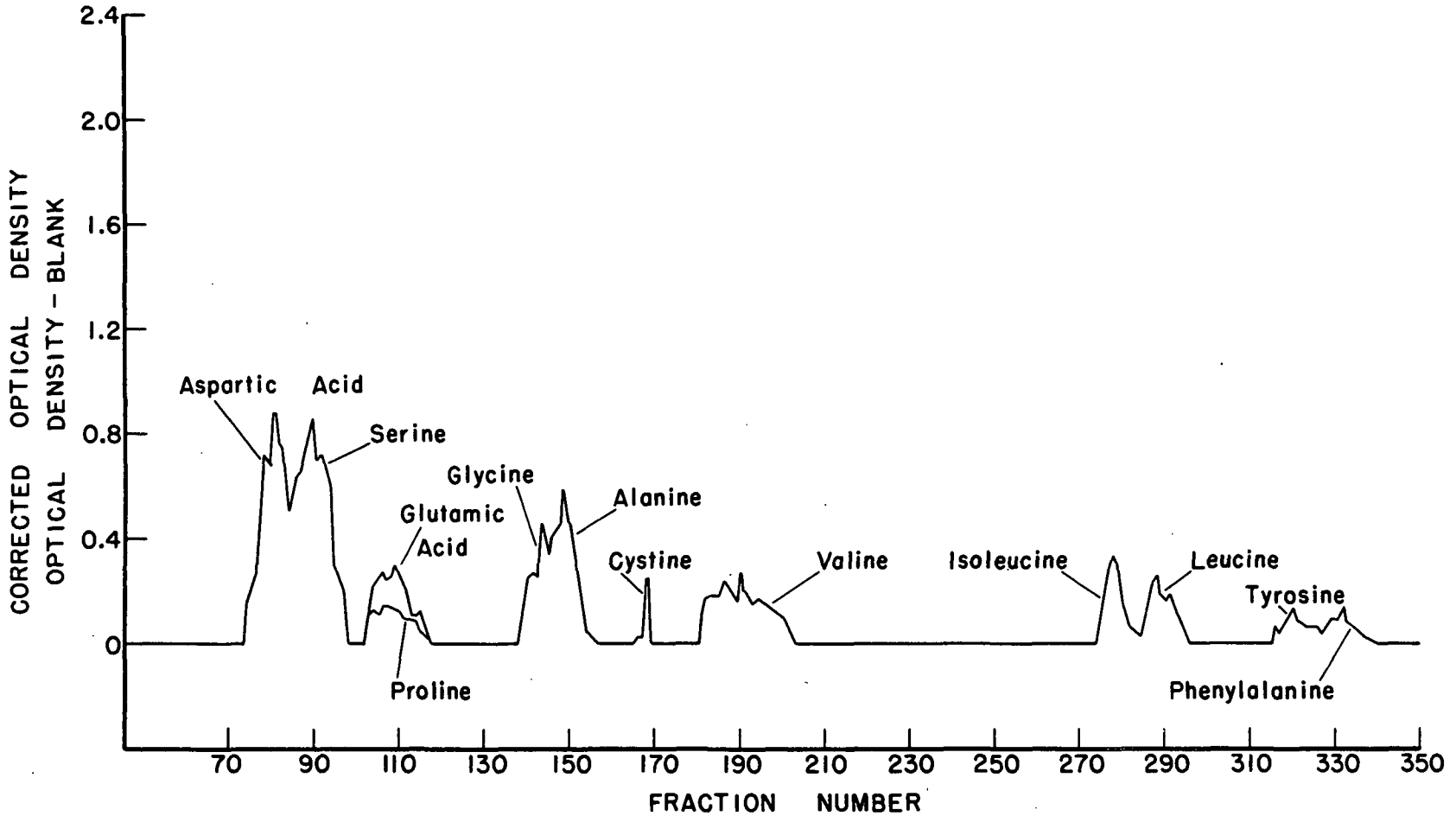


Figure 5. Identification of Amino Acids in Aspen New Xylem

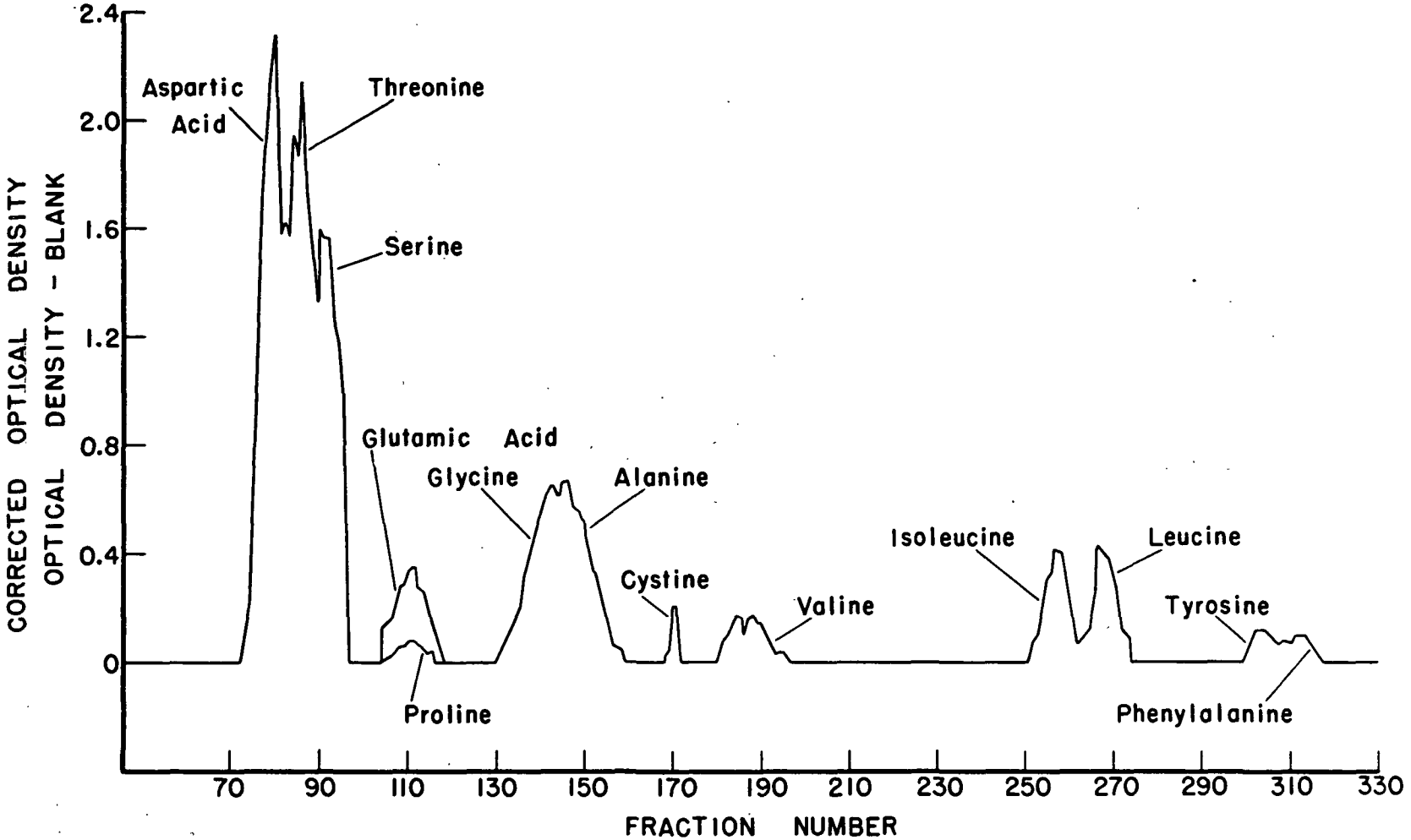


Figure 6. Identification of Amino Acids in Year-Old Aspenwood

gave a spot at 23-29 and tyrosine, a spot at 12-13. Under these chromatographic conditions, no other amino acid gives coordinates close to tyrosine (40). Accordingly, the spot at the observed coordinates was accepted as confirming the presence of tyrosine.

TABLE IX

CONCENTRATION OF ACIDIC AND NEUTRAL AMINO ACIDS IN ASPENWOOD

Compound	Concentration,[1] %	
	New Xylem	Year-Old Xylem
Aspartic Acid	0.0087	0.0022
Glutamic Acid	0.0089	0.0016
Serine	0.0089	0.0010
Proline	0.0068	0.0002
Glycine	0.0017	0.0003
Alanine	0.0031	0.0005
Threonine	0.0000	0.0009
Cystine	0.0017	0.0001
Valine	0.0042	0.0002
Isoleucine	0.0020	0.0003
Leucine	0.0022	0.0021
Tyrosine	0.0024	0.0002
Phenylalanine	0.0018	0.0001

[1] Calculations based on the ovendry, extracted wood weight.

METHANOL-SOLUBLE MATERIALS

PAPER CHROMATOGRAPHY

The methanol-soluble materials from new xylem were chromatographed on paper using the TAW, BAW, and CAW developers. In BAW and CAW, the materials moved with the solvent. In TAW, part of the materials streaked slightly from the origin but the major portion did not move. The methanol-soluble materials gave strong Maüle and Wiesner reactions.

Because of the similarity between the properties of the methanol-soluble materials and Brauns' aspen lignin, i.e., both are soluble in methanol, but insoluble in water and hexane, a solution of Brauns' aspen lignin was chromatographed beside the methanol-soluble materials. With the TAW, BAW, and CAW developers, both behaved similarly and were not distinguishable. The sample of Brauns' aspen lignin used for this and other comparisons was prepared from mature aspenwood by Leaf (54).

BRAUNS' LIGNIN

For further comparison of the methanol-soluble materials from new xylem with Brauns' aspen lignin, the methanol-soluble materials were fractionated by Brauns' procedure (55). For this, the 13 solutions of methanol-soluble materials were combined into four groups corresponding to the 1956 collection periods.

In preparing the Brauns' lignin samples, one exception to Brauns' procedure was made. Instead of precipitating the solids into anhydrous ether until a constant methoxyl content was attained, the solids were precipitated twice and then considered to be free of ether-soluble materials. The ether recovered from the second precipitations was colorless and, when evaporated, was found to have a negligible solids content.

The Brauns' lignin from the first three preparations were light tan powders which resembled quite closely in appearance the Brauns' lignin from mature aspenwood. The fourth preparation, however, dried in hard brown lumps which were later found not to be completely soluble in 95% ethanol. The reason for these differences was not known, but it was found that the

fourth sample was recovered in a much lower yield than the first three. The yields of Brauns' lignin based on the ovendry, extracted wood weight are given in Table X. The yield of Brauns' lignin from mature aspenwood was 0.7% (54).

TABLE X

BRAUNS' LIGNIN CONTENT OF ASPEN NEW XYLEM

Tissue Collection Period	Brauns Lignin Content of Tissue, %
I	0.14
II	0.23
III	0.37
IV	0.06

Determinations of the methoxyl content, infrared spectrum, and ultraviolet spectrum in neutral and alkaline solution were carried out for each preparation. Only the infrared and ultraviolet spectra of Leaf's preparation were determined since the methoxyl content had been previously reported to be 19.5% (54). Table XI gives the results of the methoxyl determinations, which were done according to Institute Method 18. (56). These results are the average of two determinations.

TABLE XI

METHOXYL CONTENT OF BRAUNS' LIGNIN FROM ASPEN NEW XYLEM

Tissue Collection Period	Methoxyl Content of Brauns' Lignin from Tissue, %
I	20.6
II	20.0
III	20.4
IV	22.8 (one determination only)

Figure 7 shows the infrared spectra of the four preparations from new xylem and of Leaf's preparation. Figure 8 shows their ultraviolet spectra in 95% ethanol solution, and Figure 9, their ultraviolet spectra in 95% ethanol containing 1.0N potassium hydroxide. Table XII gives the extinction coefficients of the Brauns' lignin preparations.

TABLE XII

EXTINCTION COEFFICIENTS OF BRAUNS' ASPEN LIGNIN

Tissue from Which Preparation Was Taken	Extinction Coefficient (ϵ)	
	At 235 mmu.	At 275 mmu.
Aspen new xylem I	38.3	20.4
Aspen new xylem II	41.4	24.8
Aspen new xylem III	50.5	29.6
Aspen new xylem IV	—	—
Mature aspenwood	48.6	29.2

PRECIPITATES FROM ETHER

Paper chromatography of the precipitates from ether revealed that they were very similar to the methanol-soluble materials; that is, in BAW and CAW the precipitates moved near the solvent front, while in TAW, most of the material stayed at the origin. Although the precipitates from ether remained in aqueous solution when the methanol was removed from the original extracts, no check was made to determine if the precipitates could be redissolved in water after being taken to dryness.

The precipitates gave strong Mäule and Wiesner reactions.

PRECIPITATES FROM WATER

The precipitates from water were shown by paper chromatography to

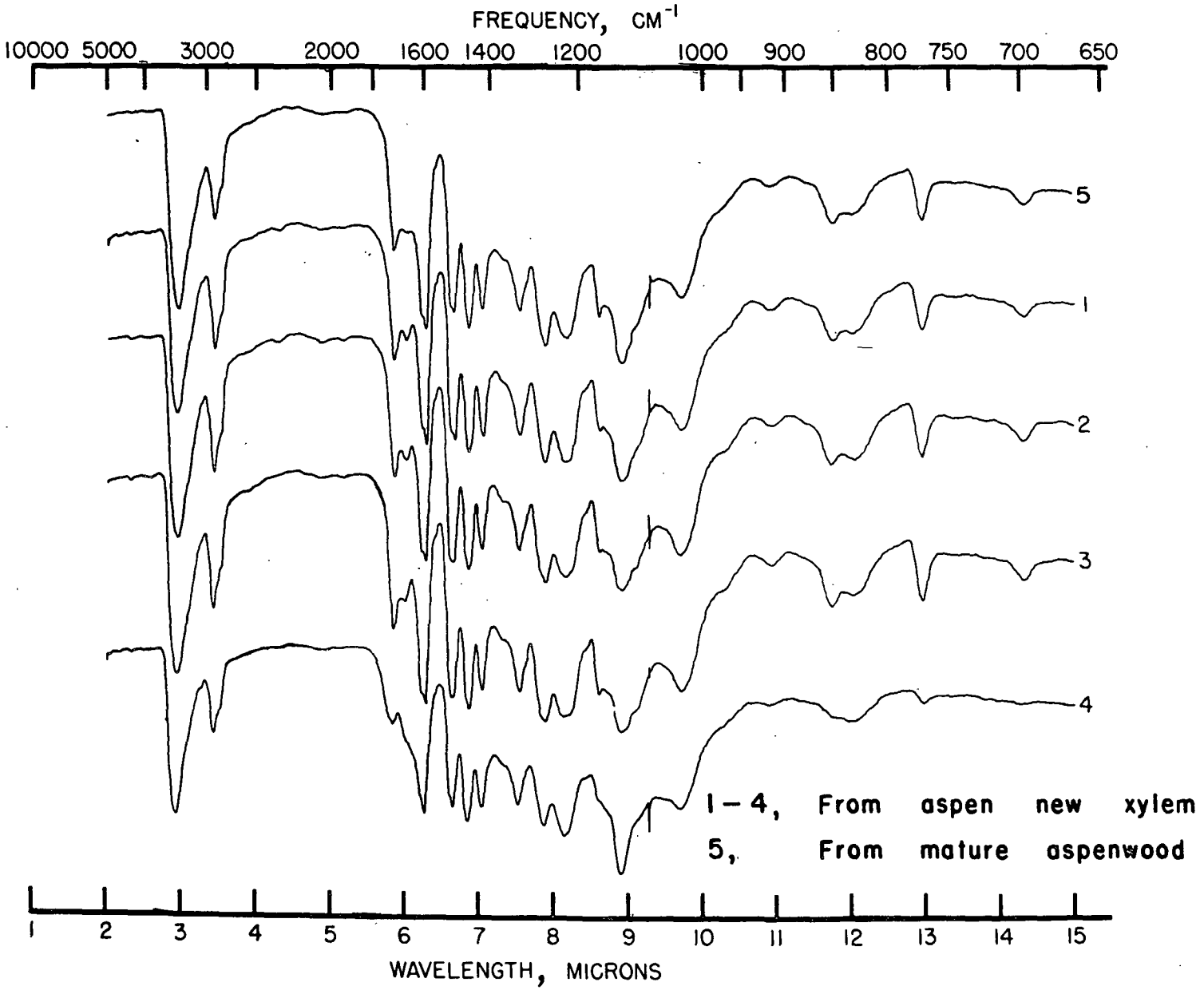


Figure 7. Infrared Spectra of Five Preparations of Brauns' Aspen Lignin

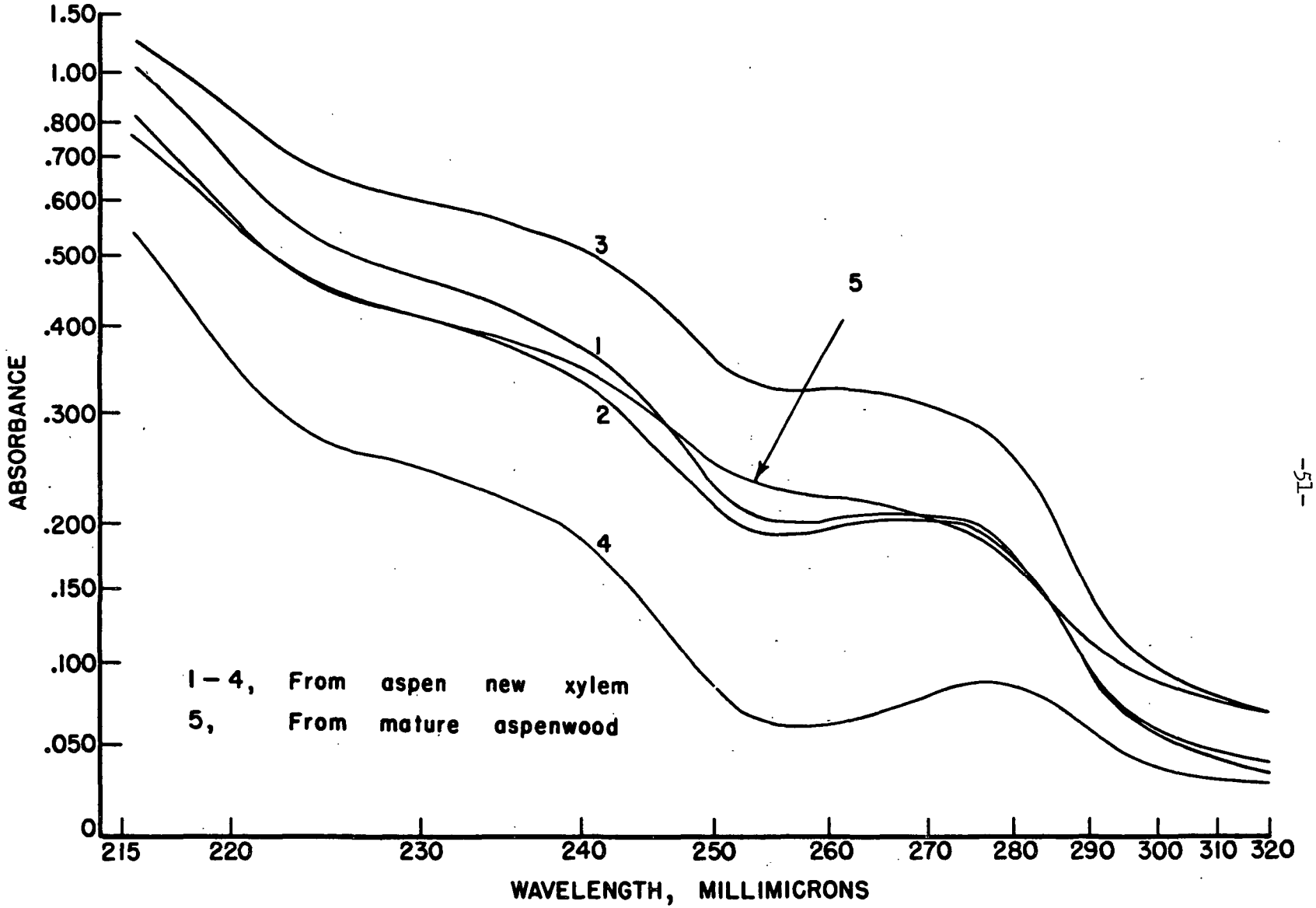


Figure 8. Ultraviolet Spectra of Five Preparations of Brauns' Aspen Lignin Solvent, 95% Ethanol

Solution Concentrations: 1, 11.75 mg./l.; 2, 9.67 mg./l.; 3, 11.84 mg./l.; 4, incompletely dissolved; 5, 8.24 mg./l.

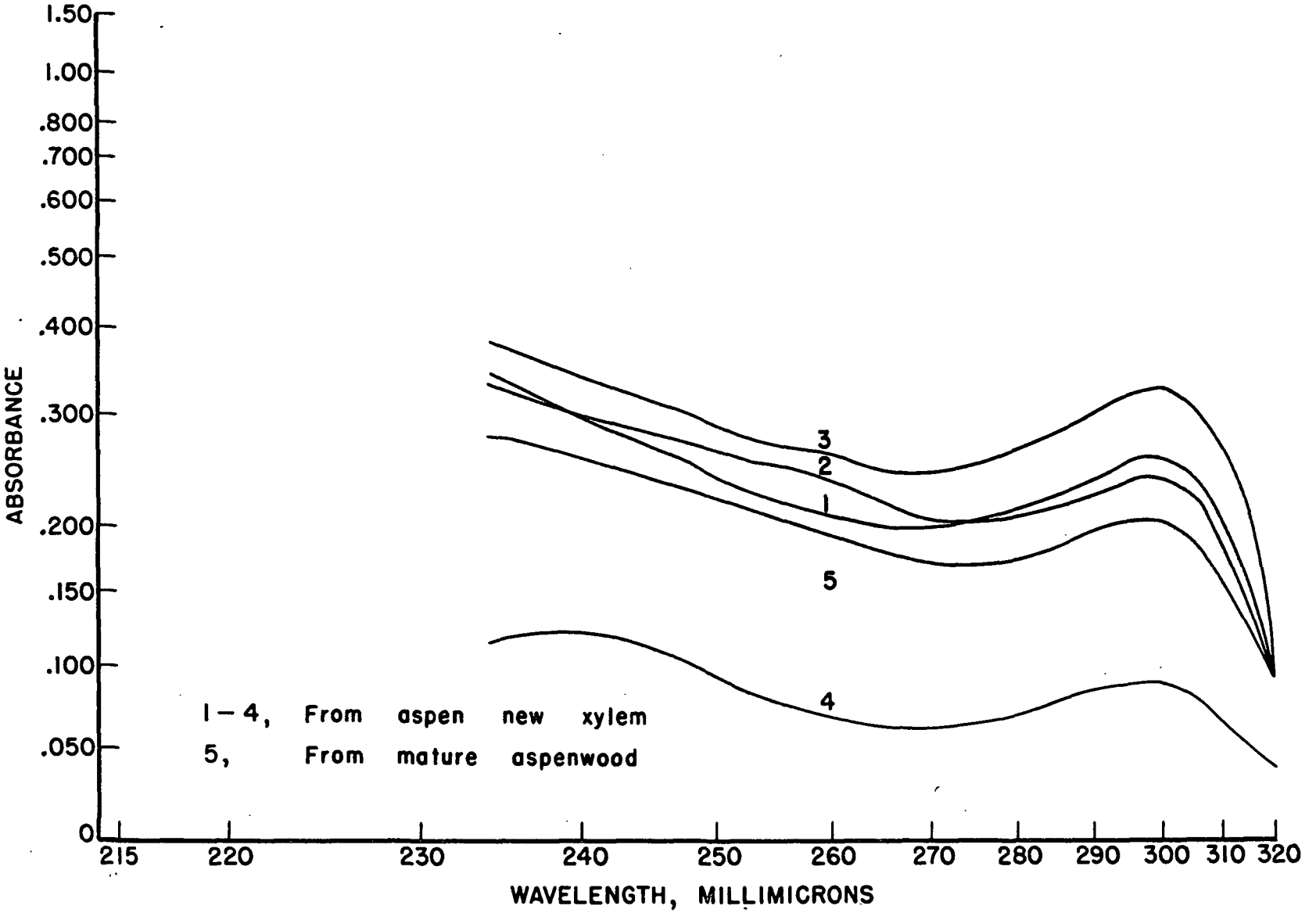


Figure 9. Ultraviolet Spectra of Five Preparations of Brauns' Aspen Lignin
Solvent, 1.0N Potassium Hydroxide in 95% Ethanol

Solution Concentrations: 1, 11.75 mg./l.; 2, 9.67 mg./l.; 3, 11.84 mg./l/;
4, incompletely dissolved; 5, 8.24 mg./l.

contain the same materials listed in Table VIII. No further study was made of these materials.

HEXANE-SOLUBLE MATERIALS

The components of the hexane-soluble materials were waxy and colorless. On paper chromatograms developed with TAW, they were found at the solvent front. With the Wiesner reagent, they gave a light pink color, which was probably due to oxidized fatty materials (70).

CHEMICAL DIFFERENCES BETWEEN ASPEN TREES

One of the stated purposes of this study was to determine if there were any variations in the composition of the methanol extractives of newly formed aspenwood which could be correlated with biometric data about the trees from which the tissue was taken. Chromatography of each fraction prepared from the methanol extracts of the new xylem of 13 *P. tremuloides* trees revealed no differences due to sex, growth site or physical condition of the tree. Comparison with extracts of the new xylem of 5 *P. grandidentata* trees showed no differences due to the difference in species.

Five *P. tremuloides* trees were found to contain at least six materials (cf. Table VIII) which were not found in the other eight trees studied, but the occurrence of these materials could not be correlated with any of the biometric data. The materials had a limited solubility in water, were very soluble in methanol and insoluble in ether and hexane.

DISCUSSION

The results of this study have shown that while some of the compounds thought to be lignin precursors do occur in newly formed aspenwood, several were not to be found there. The presence or absence of any compound cannot be taken as proof that the compound is or is not a lignin precursor, but it does give a basis for the discussion of the existing theories of lignin and lignification, and for the presentation of new hypotheses.

GENERAL METHODS

One of the uncertainties in an investigation of this nature is that the methods used may bring about changes in the extracted materials. During this study, no evidence of such changes occurring were noted, except for the following two incidents.

The fact that Compound K was extracted by methanol at room temperature or higher, but not at 0°C., indicated that the compound may be loosely combined in the original tissue.

The type of reaction which produces the artifact, Substance M, is not known. Since this material occurred only in eluates from Magnesol columns, it must be concluded that the acid-washed Magnesol catalyzes a degradation or a combination of the materials present. Hyflo Supercel was also present in the columns, but since Substance M was not formed when the ether-soluble materials were filtered through this material, it would not seem to be responsible for the reaction.

PHENYLALANINE AND TYROSINE

Phenylalanine and tyrosine, the two C_6-C_3 amino acids considered to be among the first aromatic compounds produced from carbohydrates by lignifying plants, were found to occur in significantly greater quantities in newly formed aspenwood than in mature tissue. Since protein and other nitrogenous compounds are necessary for life of any sort to exist (57), the presence of phenylalanine and tyrosine in living tissue is to be expected, and the reasons for their presence may be manifold. However, from what is known of the reactions of these compounds, it is possible to speculate about their fate in wood.

Radioactive tracer studies have shown that phenylalanine and tyrosine can be utilized by young wheat plants to produce lignin while young maple, balsam poplar, and caragana plants were able to use only phenylalanine (7). From this, there appears to be good reason to consider phenylalanine a precursor of lignin for both grasses and trees. The role of tyrosine is less apparent.

Lignin from plants of different species and families is known to vary in regard to its component groups. The lignin of grasses includes p-hydroxyphenyl, guaiacyl, and syringyl groups (7,58), while most tree lignin contains only the latter two groups (59). Because of the difference in grass lignin, one might conclude that the tyrosine which was administered to the wheat plants went primarily to p-hydroxyphenyl lignin. Unfortunately, no analysis of the C^{14} content of the p-hydroxyphenyl lignin of the wheat was made, but it was shown that an appreciable quantity of the C^{14} applied as

tyrosine was recovered as vanillin and syringaldehyde from the oxidation products of wheat (7). Whether tyrosine acts as a precursor of grass lignin only or whether some species of trees can utilize this compound is not known.

Besides their possible function as lignin precursors, the phenylalanine and tyrosine present in aspen new xylem may be acting as part of the protein cycle. Since newly formed aspenwood contains several times as much protein as mature wood does (33), most of the nitrogenous materials in the young tissue are degraded, taken from the young tissue to some other part of the tree, or converted to cell-wall material. Because of the high energy level of the aromatic nuclei of phenylalanine and tyrosine, it might be argued that they would not be synthesized if they had to be degraded before being used. However, the fact that reactions for the biochemical degradation of tyrosine to aliphatic compounds are known (61) detracts somewhat but not entirely from this argument.

It does not seem likely that the plant would remove protein from the newly formed tissue at the end of the growing season and store it in some other part of the tree, but this cannot be stated positively since little is known about the protein of trees. It has been suggested that one reason that the living plant cells cease to function might be that their protein has been used up in the formation of lignin (62). This proposal agrees well with Wardrop's hypothesis that the lignin precursors arise from within the lignifying cells (40).

SERINE AND GLYCINE

The aliphatic amino acids, serine, glycine, and methionine have been

shown to be capable of transmethylation reactions with phenols to form the methoxyl groups of lignin (26,27). Aspen new xylem was found to contain serine and glycine, but there was no indication of methionine. The quantity of serine and glycine in mature aspenwood was much lower than in the new xylem, showing that as in the case of phenylalanine and tyrosine, the nitrogenous components of newly formed tissues are either utilized in forming cell-wall materials, degraded, or transported to other parts of the tree after the growing season is over. Further studies will be necessary before serine and glycine can be regarded as sources of the methyl groups in aspen lignin, but their presence in the young tissue does show that they are available for such usage.

SINAPALDEHYDE

Two ideas have been set forth which bear on possible functions of sinapaldehyde. Adler suggested that sinapaldehyde might be an intermediate in the biosynthesis of sinapyl alcohol from phenylalanine (3). According to Freudenberg, coniferaldehyde is a "secondary building stone" of softwood lignin. Thus, sinapaldehyde, the structural analog of coniferaldehyde, might be classed as a "secondary lignin building stone."

Although there is reason to believe that coniferyl- and sinapyl alcohols are the monomers of hardwood lignin (25), little more than speculation has been offered about the way in which these compounds are biosynthesized. The suggestion that ferulic and sinapic acids are reduced to the corresponding alcohols is of interest since it parallels the known biochemical reduction of pyruvic acid to glycerol (2). In this reduction, glyceric aldehyde is an

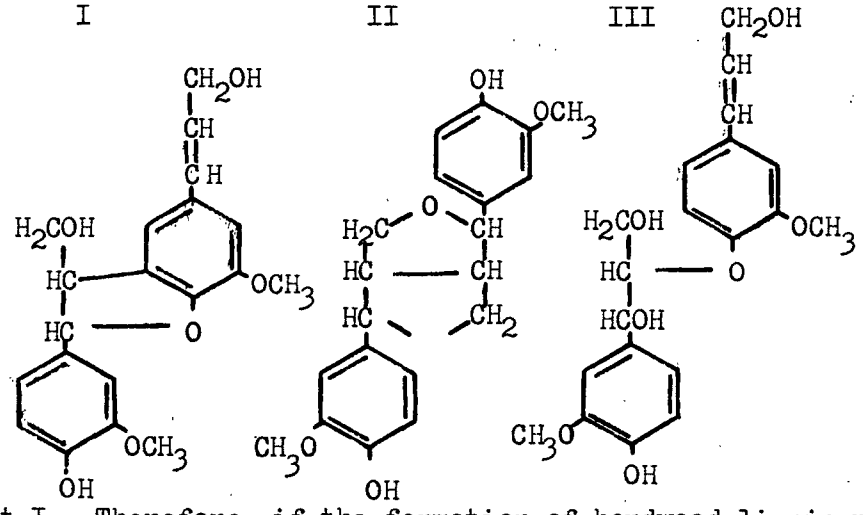
intermediate. However, if this reduction is one of the steps in the development of lignin, then sinapic acid, ferulic acid, and coniferaldehyde should be present in new xylem in addition to sinapaldehyde.

If sinapaldehyde is to be thought of as a "secondary building stone" in hardwoods, functioning in the same way as Freudenberg suggested coniferaldehyde to function in the development of soft wood lignin, then some consideration must be given to the rest of Freudenberg's theory.

Freudenberg postulates that coniferin and syringin are transported by the sap to the zone of lignification and there hydrolyzed by glucosidases. The liberated aglucones, coniferyl and sinapyl alcohol, then polymerize to form lignin. In the present study, no syringin was found in the new xylem although a very sensitive indicator was used. Neither was any evidence of coniferyl alcohol found. Thus, it may be concluded that unless the hydrolysis and polymerization reactions occur so rapidly that a detectable concentration of the reactants is never present, then Freudenberg's theory is in error. It is possible, however, that the hydrolysis and the initial stages of the polymerization take place outside of the zone of lignification, and if this possibility is accepted, a mechanism may be postulated which explains the present findings as well as most of Freudenberg's views.

Freudenberg identified three dimers of coniferyl alcohol which were isolated from spruce cambial saps (21). The structure of these compounds, dehydrodiconiferyl alcohol (I), d,l-pinoresinol (II), and guaiacylglycerol-β-coniferyl ether (III), were considered to be representative of the types of units of which soft wood lignin is composed. In the formation of hardwood

lignin, sinapyl alcohol could form dimers corresponding to II and III but



not I. Therefore, if the formation of hardwood lignin proceeds in the same general way as the formation of softwood lignin is thought to proceed, then coniferyl alcohol is necessary for structures of the same type as I to be formed. From this it would follow that coniferyl alcohol may control the lignification reactions. This would agree with the observation that sinapyl alcohol alone did not produce an insoluble polymer when treated with mushroom oxidase, but did form such a polymer when mixed with coniferyl alcohol (25).

If coniferyl alcohol is the controlling factor in the lignification of hardwoods, and if there is an excess of sinapyl alcohol present to react with the coniferyl alcohol, then the coniferyl alcohol could be expected to polymerize as soon as it is formed. Thus, at any time, there would be an excess of sinapyl alcohol present but only a slight amount of coniferyl alcohol. If the enzymes present in the zone of lignification are comparable to the mushroom enzymes used by Freudenberg, the excess alcohol could be

oxidized to sinapaldehyde. The fact that only small amounts of coniferaldehyde were found with the three dimers of coniferyl alcohol (22), may indicate that the compound was formed by a side reaction such as the oxidation of an excess of the alcohol. The fact that aspenwood contains more syringyl than guaiacyl groups (63) might indicate that more sinapyl than coniferyl alcohol was present during lignification.

Although sinapaldehyde may be the oxidation product of an excess of sinapyl alcohol, it should not be thought of as a metabolic waste material. The difference in the concentration of the aldehyde in new xylem and mature wood shows that it is being used by the tree in some way. The presence of coniferaldehyde and/or sinapaldehyde structures in aspen lignin as revealed by its positive Wiesner reaction indicates one way in which the compound may be used. Because the sinapaldehyde would probably be combined with the lignin chiefly as an end group, there may be insufficient sites for its complete utilization, hence the tree sinapaldehyde found in mature aspenwood.

Both coniferaldehyde and sinapaldehyde have been isolated from the hydrolysis products of extracted aspenwood (60). Therefore, it is possible that both compounds are linked to lignin. If this is true, then it must be assumed that the coniferaldehyde is combined much more completely than sinapaldehyde.

BRAUNS' LIGNIN

Comparison of Brauns' lignin isolated from newly formed aspenwood, which is incompletely lignified, with Brauns' lignin from mature aspenwood showed

that there was a great similarity between the two materials. The significance of this similarity cannot be accurately assessed since the true nature of Brauns' lignin is not known. However, some of the results of the present study do suggest one possibility about the composition of Brauns' lignin.

From the similarity of the chromatographic properties of the precipitates from ether and Brauns' lignin (cf. pp. 47, 49) appear that both materials had the same general composition, but from the difference in their solubility properties, there is evidently considerable difference in their molecular size. Thus, it may be that both are composed chiefly of incompletely polymerized lignin building stones. A related view was given by Freudenberg who stated that lignin exists in several condensation stages (64). The failure of these materials to attain the higher molecular weight of insoluble lignin could be due to end group reactions such as occur in many polymerizations, thereby producing a wide assortment of molecular sizes in the final product.

The suggestion has been made that aspen lignin may be significantly different from other hardwood lignin (65). One point on which this could be checked would be the comparison of the infrared spectrum of Brauns' aspen lignin with the spectra of Brauns' lignin from other hardwoods. The most applicable spectra for such a comparison appear to be those reported for Brauns' birch, oak, and maple lignin by Nord and Schubert (66). For further information, the spectra of the Brauns' lignin of the four hardwoods was compared with the spectra of Brauns' spruce lignin and Brauns' slash pine lignin obtained by Lyness and Schenker (67), and the spectrum of Brauns' white Scots pine lignin determined by Nord and Schubert (66).

In general, all of the spectra showed the same absorption bands, but distinct differences were observed in the relative intensities of the hydroxyl and aliphatic carbon absorption bands. In the spectra of the Brauns' lignin of aspen, slash pine, and black spruce, the hydroxyl band (3.0 mu.) was much stronger than the aliphatic carbon band (3.4 mu.) On the contrary, in the spectra of the Brauns' lignin from birch, oak, maple, and white Scots pine, the aliphatic carbon band was much stronger than the hydroxyl band.

It is unfortunate that these data are so limited as to the number of species, and the generally similar spectra too divergent in the relative intensity of these two important absorption bands to permit conclusions to be drawn. It is to be hoped that additional determinations will clarify this subject.

PARA-HYDROXYBENZOIC ACID

Para-hydroxybenzoic acid has been found free in aspenwood (65) and has been shown to be a constituent of the cell wall of aspenwood (60) and of Brauns' aspen lignin (68). In the present study, the free acid was found in the youngest, unlignified tissue of aspen, the xylem scrapings. Because this compound has been found only in trees of the Populus genus and the closely related Salix genus (63), and because p-hydroxyphenyl units have previously been found in significant quantity only in the lignin of grasses (58), the presence of p-hydroxybenzoic acid in aspenwood is of much interest, although its function is not known. The acid has been found to be capable of acting as a precursor of lignin (7), but the relatively large quantity of it which is present in aspenwood does not indicate per se that it functions in this way.

CONCLUSIONS

From the results of this study, the following conclusions may be drawn:

1. Phenylalanine and tyrosine are available in aspen new xylem for conversion to lignin. The amounts present in the new xylem are, tyrosine 0.0024%, and phenylalanine, 0.0018%; in year-old xylem, 0.0002 and 0.0001%, respectively.

2. Serine and glycine are available in aspen new xylem for transmethylation reactions with phenolic hydroxyls to form methoxyl groups.

3. Free sinapaldehyde was found in newly formed and mature aspenwood.

4. The average concentration of free sinapaldehyde in aspen new xylem, 0.063%, is five to eight times greater than its concentration in mature wood.

5. Nine (or more) water-soluble materials which give the Mäule and/or Wiesner reactions and which may represent partially polymerized lignin building stones, occur throughout the bole of an aspen tree.

6. Shikimic acid, ferulic acid, sinapic acid, coniferyl alcohol, coniferaldehyde, and syringin were not found in aspen new xylem.

7. A material corresponding to Brauns' aspen lignin was found in aspen new xylem collected in late June, i.e., 6-8 weeks after the start of the growing season. Accordingly, the material was present soon after the deposition of lignin began.

8. Para-hydroxybenzoic acid is present in the youngest, unlignified tissues of aspenwood as well as in more mature tissue.

9. With the available data, differences between the infrared spectrum of Brauns' aspen lignin and the infrared spectra of three other hardwoods and three softwoods cannot be correlated with the botanical subdivisions to which the trees belong.

10. Populus tremuloides trees of different sex, growth site, and physical condition were not distinguished by the composition of the methanol extracts of their xylem.

11. Populus tremuloides and Populus grandidentata trees were not distinguished by the composition of the methanol extracts of their xylem.

12. Contacting the water-soluble, ether-soluble materials of aspenwood with acid-washed Magnesol produced a Wiesner-positive artifact.

13. Extraction of aspenwood with methanol at 20°C. or higher removes a Wiesner-positive material which is not extracted by methanol at 0°C.

GLOSSARY

1. Definition of Terms Used:

   (a) phloem--inner living bark.

   (b) xylem scrapings--(XSc), unlignified newly formed tissue composed of the cambium and a few layers of xylem cells adjacent to the cambium.

   (c) soft xylem--(SX), newly formed tissue containing 5-10% lignin.

   (d) new xylem--(NX), newly formed tissue containing 13-19% lignin.

   (e) Wiesner-positive--giving a red-violet color when treated with the Wiesner reagent (1% phloroglucinol in 12% hydrochloric acid solution).

   (f) Maüle-positive--giving a red color when subjected to the Maüle test (chlorination followed by contacting with sodium sulfite solution).

   (g) M-W materials--ether-soluble materials from aspen xylem which give positive Maüle and/or Wiesner reactions.

2. Paper Chromatographic Developers:

   (a) TAW, toluene-acetic acid-water (4:1:5).

   (b) BAW, butanol-acetic acid-water (63:10:27).

   (c) BWZ, butanol-water-acetic acid (4:5:1).

   (d) CAW, meta cresol-acetic acid-water (50:2:48).

   (e) NBEW, n-butyl ether saturated with water.

   (f) BF, benzene saturated with formic acid.

   (g) PFW, phenol-formic acid-water (3:0.01:1).

   (h) BAm, butanol-2% ammonia (by weight).

(i) BKWAm, butanol-methyl ethyl ketone-water-17N ammonium hydroxide (5:3:1:1).

3. Indicators for Materials on Paper Chromatograms:

(a) Wiesner reagent, 1% phloroglucinol in 12% hydrochloric acid solution.

(b) Mäule test, chlorination of a damp chromatogram followed by spraying with sodium sulfite solution.

(c) DNPH, 2,4-dinitrophenylhydrazine in hydrochloric acid solution.

(d) BDB, bis-diazotized benzidene.

(e) p-Anisidine, p-anisidine hydrochloride in butanol solution.

(f) Ninhydrin, ninhydrin in butanol solution.

(g) Periodate-piperazine-nitroprusside reagents, sodium periodate solution and piperazine-sodium nitroprusside solution used in that order to spray a chromatogram.

(h) Propanol-hydrochloric acid, n-propanol containing 5% concentrated hydrochloric acid.

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Charts are presented which outline many of the reactions and reaction sequences which are known to occur in living plant tissue. One sequence is given by which pyruvic acid is reduced to glycerol. Glyceric aldehyde is one of the intermediate products of the sequence.

3. Adler, E., Tappi 40, no. 4:294-301(April, 1957).

The present views and recent experimental work are summarized. The article reviews the work and theories of

(a) Davis (5)

(b) Brown and Neish (7, 8)

(c) Ehrensvärd and co-workers (6)

(d) Byerrum and co-workers (26, 27)

(e) Eberhardt and Schubert (9)

(f) Freudenberg (19-23, 25).

From this, an outline of a pathway by which lignin may be formed is presented. According to this pathway coniferyl and sinapyl alcohol, the suggested monomers of lignin are formed from glucose. Intermediate compounds include 5-dehydroquinic acid, 5-dehydroshikimic acid, shikimic acid, prephenic acid, phenylpyruvic acid, p-hydroxyphenylpyruvic acid, phenylalanine, tyrosine, cinnamic acid, and ferulic and sinapic acids.

4. Calvin, M. The path of carbon in photosynthesis. Reilly Lectures, Vol. II. Notre Dame, Indiana, The University of Notre Dame, 1949.

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Growth studies of the bacterial mutant E. coli have established that this mutant is capable of synthesizing phenylalanine from glucose. Intermediate compounds in their order of formation were found to be 5-dehydroquinic acid, 5-dehydroshikimic acid, shikimic acid, and phenylpyruvic acid. Prephenic acid was found to be inactive as a nutrient but was assigned a position in the pathway because it was found to be converted to phenylpyruvic acid by the mutant.

6. Tatum, E. L., Gross, S. R., Ehrensvärd, G., and Garnjobst, L., Proc. Natl. Acad. Sci. U. S. 40:271-6(1954).

Metabolic reactions of a mutant of the fungus Neurospora crassa were studied. Beginning with labeled glucose, it was shown that all of the carbon atoms of the aromatic nucleus of tyrosine were derived from shikimic acid.

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Compounds labeled with C^{14} were fed to wheat, maple, balsam poplar, and caragana plants. After 24-48 hours the plants were extracted and the cell-wall materials oxidized. The ratio of the quantity of C^{14} fed to the plants and the quantity recovered as aromatic aldehyde was

determined, and termed the efficiency of conversion to lignin. Phenylalanine, tyrosine, ferulic acid, cinnamic acid, and vanillin were converted to wheat lignin with high efficiencies. Para-hydroxybenzoic acid and phenylpyruvic acid were converted with lower but significant efficiencies. Benzoic acid, anisic acid, vanillic acid, protocatechuic acid, trimethyl gallic acid, syringic acid, and acetaminocinnamic acid were not efficiently converted to wheat lignin. The maple, balsam poplar, and caragana plants converted phenylalanine efficiently but not protocatechuic acid or tyrosine.

8. Brown, S. A., and Neish, A. C., Nature 175:688-9(1955).

Labeled shikimic acid was fed to wheat and maple plants. A high proportion of the C^{14} was recovered as vanillin and syringaldehyde when the extracted tissue was oxidized with nitrobenzene.

9. Eberhardt, G., and Schubert, W. J., J. Am. Chem. Soc. 78:2835-7(1956).

Shikimic acid labeled with C^{14} in the 2 and 6 position was fed to sugar cane. Vanillin obtained from the nitrobenzene oxidation of the cuoxam lignin isolated from the cane showed a distribution of radioactivity comparable to the distribution in the shikimic acid.

10. Hasegawa, M., Yoshida, S. and Nakegawa, T., Kagaku 24:421(1954); cf. Nature 175:688(1955).

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11. Anet, E. F. J. L., Birch, L. S., and Massey-Westropp, R. A., Australian J. Chem. 10:93-4(1957).

Young leaves of Eucalyptus citriodora were extracted with 95% ethanol. Shikimic acid was isolated from the extract by displacement

chromatography after the acidic materials were sorbed on Amberlite IR-4B ion-exchange resin.

12. Whiting, G. C., Nature 179:531(1957).

Shikimic acid was isolated from unripe gooseberry fruit by aqueous extraction. It was also found in grasses, apples, blackberries, pears, and quince fruit.

13. Goldschmid, O. Personal communication, 1957.

Goldschmid mentioned that esters of quinic and shikimic acid, not yet fully characterized, were found in cambial extracts of Western hemlock.

14. Synge, RLM. In Paech and Tracey's Modern methods of plant analysis. Vol. IV. p. 1-22. Berlin, Springer-Verlag, 1955.

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19. Freudenberg, K., Mitt. d. Osterr. Gesellschaft f. Holzforschung no. 3:72-3(June, 1952).

Softwood lignin is stated to be the polymerization product of a radical of coniferyl alcohol. Coniferyl alcohol is said to come to the lignifying tissue as coniferin and be liberated there by glucosidic enzymes, which exist only in the narrow region just inside the cambium. The coniferin is said to be produced elsewhere and brought in by the tree sap.

Hardwood lignin is stated to be a mixed polymer of coniferyl and sinapyl alcohols.

20. Freudenberg, K. and Heimberger, W., Chem. Ber. 83:519-30(1950).

Treating free coniferyl alcohol with phenoldehydrogenase produced a dehydrogenated polymer (DHP) identical with or closely resembling spruce lignin. The infrared and ultraviolet spectra of the two materials were not distinguishable. Both are soluble in bisulfite solutions and the DHP yielded a lignosulfonate having the same composition as spruce lignosulfonate. The DHP had a molecular weight of 810-820 (by isothermal distillation), gave the lignin color reactions, was optically inactive, and gave 95.5% Klason lignin. Spruce and DHP thioglycolic lignin were found to be similar.

21. Freudenberg, K., and Schluter, H. Chem. Ber. 88:617-25(1955).

On enzymatic dehydrogenation of coniferyl alcohol, dimeric intermediates formed. These were dehydrodiconiferyl alcohol, pinoresinol, guaiacylglycerol-β-coniferyl ether, and coniferaldehyde.

22. Adler, E., Tappi 40, no. 4:294-301(1957).

Adler reports Freudenberg's findings concerning the per cent of each "secondary lignin building stone" formed when coniferyl alcohol is reacted with mushroom oxidase. Depending upon the condition these values were:

(a) dehydrodiconiferyl alcohol, 60%, 20%

(b) d,l-pinoresinol 25%, 10%

(c) guaiacylglycerol-β-coniferyl ether 15%, 70%

(d) coniferaldehyde, less than 1% in either case.

Adler reports an experiment of Freudenberg's in which radioactive phenylalanine and ferulic acid were fed to young spruce plants. Ethanolysis of the spruce lignin yielded radioactive "Hibbert ketones." Adler believes that the formation of the ketones is due to the presence of guaiacylglycerol-β-aryl ether groups in lignin. Therefore, it is concluded that the carboxyl groups of the two acids administered to the plants were reduced to primary alcohol groups.

23. Freudenberg, K. Personal communication to H. F. Lewis, 1956.

Dr. Freudenberg stated that pinoresinol, dehydrodiconiferyl alcohol, guaiacylglycerol-β-coniferyl ether and coniferaldehyde had been found in spruce sap.

24. Kratzl, K. and Billek, G., Tappi 40, no. 4:269-85(April, 1957).

A small quantity of a dehydrogenation polymer (DHP) of sinapyl alcohol was formed by treating the alcohol with mushroom dehydrogenase. Ethanolysis of the DHP produced "Hibberts ketones". From this it was concluded that sinapyl alcohol will form dimers of the β-ether type.

The formation of "Hibberts ketones," (arylpropane-ketones) upon ethanolysis is stated as one of eight "lignin criteria," which distinguish lignin from other wood substances.

25. Freudenberg, K. and Hubner, H. H., Chem. Ber. 85:1181-91(1952).

A dehydrogenation polymer (DHP) of a mixture of equal parts of coniferyl and sinapyl alcohol and approximately the same carbon, hydrogen, and oxygen content as Beech cuoxam lignin. Sinapyl alcohol alone does not yield a satisfactory dehydrogenation polymer with mushroom enzymes. A large portion remains in solution.

The 2,4-dinitrophenyl ether of dehydrodiconiferyl alcohol was prepared by reacting the alcohol with 1-fluoro-2,4-dinitrobenzene in the presence of sodium bicarbonate.

26. Byerrum, R. V., Flokstra, J. H., Dewey, L. J., and Ball, C. D., J. Biol. Chem. 210:633-43(1954).

Methionine labeled with C^{14} was fed to barley and tobacco plants. By the method of (27) the radioactivity of the methoxyl groups of the barley and tobacco lignin was determined. From this it was found that the methyl group of methionine is transferred as a unit to form the methoxyl groups of lignin in barley and tobacco.

27. Hamill, R. L., Byerrum, R. V., and Ball, C. D., J. Biol. Chem. 224:713-16(1957).

Serine -3-C^{14}, and glycine-2-C^{14}, and glycine-1-C^{14} were fed to tobacco plants. After 7 days, the plants were cut and extracted with hydrochloric acid to remove nitrogenous materials. The C^{14} content of the unextracted methoxyl groups was determined by counting the radioactivity of the methyltriethylammonium iodide formed when triethylamine was reacted with the methyl iodide produced by demethoxylation of the lignin with hydroiodic acid. The β-carbon of serine is introduced in lignin to the greatest extent. The α-carbon of glycine was incorporated in about one-third the quantity of the β-carbon of serine, whereas the glycine carboxyl group was introduced in about one-tenth the quantity of the β-carbon of serine.

28. Kremers, R. E., Tappi 40, no. 4:262-8(April, 1957).

A tabulation of the known occurrence of thirteen aromatic compounds which may be intermediates in the biosynthesis of lignin is presented.

A photomicrograph of xylem scrapings taken from p. tremuloides is presented.

29. Brauns, F. E. The chemistry of lignin. New York, Academic Press, Inc., 1952. 808 p.

Several theories on the formation of lignin are reviewed. Materials mentioned as lignin precursors include cellulose, pentosans, pectins, water-soluble sugars, and olivil resins.

30. Siegel, S. M., Quart. Rev. Biol. 31:1-12(1956).

Several theories concerning the formation of lignin are reviewed. Compounds suggested as precursors include benzene carboxylic acids, coniferaldehyde, hydroxy-coniferaldehyde, and 1-hydroxy-2-keto-3-phenyl propane.

31. Wardrop, A. B., Tappi 40, no. 4:225-43(April, 1957).

Experiments are described in which Pinus radiata and Eucalyptus cladocalyx stems were partially or completely ring barked. The patterns of cell differentiation and lignification were altered, but because lignification was not halted completely by the ring barking, it was concluded that the precursors of lignin originate within individual cells at a particular stage of their differentiation.

Experimental work bearing on the source of lignin precursors is reviewed and discussed.

32. Freudenberg, K., Reznik, H., Boesenberg, H., and Rasenack, D., Chem. Ber. 85:641-7(1952).

Syringin and coniferin are stated to come from unknown sources to the tissues being lignified. It was conceded that the aglucones might be produced at the site of lignification but this explanation was thought to be less probable than the first because the lignifying tissues were not considered to have the capacity for biosynthesis.

33. Sultze, R. F. A study of the phenolic and carbohydrate materials in the newly formed tissues of aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1956. 121 p.

Eleven Populus tremuloides trees were cut at four evenly spaced intervals during the growing season. Samples of xylem representing four stages of lignification were collected. These were xylem scrapings, soft xylem, new xylem, and year-old xylem. Procedures for collecting the tissues are described. As soon as collected, all tissue was placed immediately in methanol to stop enzyme activity. The methanol was decanted three times and fresh methanol added so that a

sixteen-day extraction period (four 4-day extractions) was effected. Afterward the extracts were concentrated and the methanol removed under vacuum. The aqueous solution and precipitated material which resulted were fractionated into the water-soluble, hexane-soluble, and methanol-soluble materials. The per cent of the methanol extract represented by each of the fractions prepared was reported.

The acidic water-soluble materials were isolated by sorption on Amberlite IR-4B resin followed by elution with dilute sulfuric acid. Paper chromatography of the acidic water-soluble materials using a butanol-water-acetic acid (4:5:1) developer produced two Maüle-positive and one Maüle-Wiesner positive spot.

Analysis of the extracted marcs showed that based on the ovendry, ash free, unextracted tissue,

(a) the xylem scrapings contained 27.3% protein and were unlignified,

(b) the soft xylem contained 8.8% protein and 6.4% lignin,

(c) the new xylem contained 1.7% protein and 16.6% lignin,

(d) the year-old xylem contained 0.6% protein and 21.3% lignin.

Comparison of the composition of year-old xylem with that reported for mature aspenwood lead to the conclusion that cell differentiation was completed in one year.

34. Kremers, R. E. Personal communication. 1956.

Dr. Kremers emphasized the need for information about individual variations between trees.

35. Bate-Smith, E. C., Sci. Proc. Roy. Dublin Soc. 27:165(1956).

The use of the upper phase of a mixture of toluene-acetic acid-water (4:1:5) for separating phenols by paper chromatography is described.

36. Reeder, B. Personal communication, 1957.

The upper phase of a mixture of butanol-acetic acid-water (63:10:77) was described as useful in resolving amino acids on paper chromatograms.

37. Stone, J., and Blundell, M., Anal. Chem. 23, no. 5:771-4(May, 1951).

A solvent composed of n-butyl ether saturated with water was used to resolve aromatic aldehydes by paper chromatography.

38. Bray, H. G., Lake, H. J., Thorpe, W. V., and White, K., Biochem. J. 47:xiii(1950).

Benzene saturated with 100% formic acid is described as a good developer for hydroxybenzoic acids on paper chromatograms.

39. Stark, J. B., Goodban, A. E., and Owens, H. S., Anal. Chem. 23, no. 3: 413-15(March, 1951).

Paper chromatography of organic acids with a phenol-water-formic acid (3:1:0.01) developer is described.

40. Wolfe, M., Biochem. et Biophys. Acta 23:186-92(1952).

Developers for two-dimensional paper chromatography of amino acids are described. These were (1) the upper phase of a mixture of butanol-acetic acid-water (4:1:5), and (2) butanol-methyl ethyl ketone-water-17N ammonium hydroxide (5:3:1:1). A diagram was given showing the location of seventeen amino acids on a two-dimensional paper chromatogram developed with the two solvent mixtures.

41. Gailey, W. R., Chemist Analyst 39:59-62(1950).

Vanillin and ethyl vanillin were separated on paper chromatograms developed with butanol containing 2% ammonia (by weight).

42. Geissman, T. A. In Paech and Tracey's Modern methods of plant analysis. Vol. III. p. 450-98. Berlin, Springer-Verlag, 1955.

Paper chromatography of phenolic materials with the lower phase of a mixture of meta cresol-acetic acid-water (50:2:48) is described.

43. Pearl, I. A. Personal communication, 1957.

Tests of a number of aromatic aldehydes showed that only coniferaldehyde and sinapaldehyde gave a red-violet color with the Wiesner reagent.

44. Campbell, W. G., McGowan, J. C., and Bryant, S. A., Biochem. J. 32:2138-9(1938).

Experiments were made which showed that the chlorine-sodium sulfite color test is specific for pyrogallol groups.

45. Hirs, C. H. W., Moore, S., and Stein, W. H., J. Biol. Chem. 195:669-83 (1951).

A spray reagent for amino acids on paper chromatograms is described. After spraying a sheet with a 2% solution of ninhydrin in butanol containing 10% acetic acid, the sheet must be heated at 100°C. for several minutes in order for the characteristic blue color to develop.

46. Hough, L., Jones, J. K. N., and Wadman, W. J., Chem. Soc. 1950:1702-6.

Para-anisidine hydrochloride was prepared by dissolving 12.3 grams of p-anisidine in 40 ml. of ethanol, and adding 10 ml. of hydrochloric

acid. After precipitating the salt with ether, it was washed with ether and air dried. A 0.5% solution of the salt in butanol was used to spray paper chromatograms, which were then heated at 100°C. for several minutes to develop the color given by aldoses.

47. Cartwright, R. A., and Roberts, E. A. H., Chem.& Ind. (London) 1955:230-1.

Quinic acid on a paper chromatogram gave a yellow-green color when sprayed with a saturated solution of sodium metaperiodate diluted with two volumes of water, air dried, and then sprayed with sodium nitroprusside (50 mg.) and piperazine (50 mg.) in 80% ethanol, and finally heated for 5 minutes at 100°C.

48. Manson, D. W. Personal communication, 1957.

d-Catechol and other compounds thought to be leucoanthocyanins gave a red color when chromatographed on paper and sprayed with propanol containing 6% hydrochloric acid.

49. Pearl, I. A., and Dickey, E. E., J. Am. Chem. Soc. 73:863-4(1951).

Syringaldehyde and vanillin were separated by chromatography on a column of acid-washed Magnesol-Celite No. 535 (5:1) developed with petroleum ether (65-110°C.)-ethanol (50:1). The Magnesol mixture was prepared by slurrying the materials in dilute hydrochloric acid, then washing them free of chloride ions, followed by overnight air drying and 18 hours of drying at 100°C. The aldehydes were absorbed on the column from benzene solution and eluted from the extruded column with acetone.

50. Lemon, H. W., Anal. Chem. 19, no. 11:846-9(Nov., 1947).

Purification of 95% ethanol for use as a solvent in ultraviolet spectrum determinations is described. The alcohol was refluxed with zinc dust and potassium hydroxide before distilling from all-glass equipment.

51. Pearl, I. A. Personal communication, 1957.

Coniferyl alcohol was stated to have an R_f value of 0.79 when chromatographed on paper using a butanol-2% ammonia developer.

52. Moore, S., and Stein, W. H., J. Biol. Chem. 192:663-81(1952).

A procedure is described for the chromatographic fractionation of mixtures of acidic and neutral amino acids on columns of Dowex-50 ion-exchange resin, sodium form. Elution of the amino acids was effected by use of buffers of progressively increasing pH, from 3.4-4.25, and increasing the temperature of the water in the column jacket from 37.5 to 75°C. The buffer system yields an effluent curve in which every component emerges as a distinct peak. Between 350-375 ml. of buffer solution are put through the column and collected in 1-ml. fractions. After ninhydrin has been added to the fractions, they are placed in a 100°C. bath for 20 minutes and then diluted with a known amount of 50% propanol.

The amino acids are identified from the position of the peaks on a plot of the fraction number versus the optical density at 570 mmu. of each fraction. The optical density of fractions thought to contain proline and hydroxyproline are checked at 440 mmu.

Recovery of amino acids is quantitative (100±3%).

53. Dubey, G. A. Personal communication, 1957.

Known amino acids were separated by the method of Moore and Stein and standard curves of their occurrence in the column effluent fractions were drawn. The optical density-concentration factor for solutions of amino acids containing ninhydrin were found.

54. Brauns, F. E., Buchanan, M. A., and Leaf, R. L., J. Am. Chem. Soc. 71:1297-9(1949).

Brauns' lignin was prepared from aspenwood. Its methoxyl content was 19.5%.

55. Brauns, F. E., J. Am. Chem. Soc. 61:2120-7(1939).

The procedure for preparing Brauns' (native) lignin is given. After extracting wood meal with water and ether, it was percolated with alcohol at room temperature. The alcohol solution is removed and the alcohol distilled off under vacuum. The precipitate is washed with water and ether, filtered and dissolved in dioxane. Then the dioxane solution is filtered and poured into water. The precipitate is removed by centrifuging. The precipitation is repeated using ether until the precipitate has a constant methoxyl value. It is then washed with high-boiling and low-boiling petroleum ether, air dried for 4 hours and dried in an Abderhalden drier at 100°C. for 3 hours.

56. Institute Method 18. Appleton, Wis. The Institute of Paper Chemistry, 1951.

To determine the methoxyl content of a material, it is digested in a hydroiodic acid - phenol-hypophosphorous acid mixture. The alkyl iodide formed is swept from the reaction flask with carbon dioxide.

The gas stream is passed through a bromine-potassium acetate-acetic acid solution. At the completion of the digestion excess bromine in the receiver is destroyed with formic acid and the iodine liberated upon addition of acidified potassium iodide is then titrated with sodium thiosulfate.

57. Fruton, J. S., and Simmonds, S. General biochemistry. New York, John Wiley and Sons, Inc., 1953. 940 p.

A quotation from Mulder's General physiological chemistry published in 1844-51 is given:

"It (protein) is without doubt the most important of the known components of living matter, and it would appear that, without it, life would not be possible."

58. Creighton, R. H. J., and Hibbert, H., J. Am. Chem. Soc. 66:37-8(1944).

Alkaline nitrobenzene oxidation of cornstalks yielded vanillin, syringaldehyde and p-hydroxybenzaldehyde.

59. Creighton, R. H. J., Gibbs, R. D., and Hibbert, H., J. Am. Chem. Soc. 66:32-7(1944).

Alkaline nitrobenzene oxidation of 47 gymnosperms and angiosperms showed that in practically all cases the former yielded only vanillin and the latter both vanillin and syringaldehyde.

60. Stanek, D. A. A study of the low-molecular weight phenols formed on the hydrolysis of aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1957. 43 p.

Aqueous hydrolysis of extracted sawdust from a Populus tremuloides tree yielded sinapaldehyde, coniferaldehyde, p-hydroxybenzoic acid, [illegible] and several other compounds.

61. Umbreit, W. W. Metabolic maps. Minneapolis, Minnesota, Burgess Publishing Co., 1952. 439 p.

Metabolic reactions from the oxidation of tyrosine to fumaric and acetoacetic acid are given. Metabolism of tyrosine by rat liver slices resulted in the formation of the two compounds.

62. Dillingham, E. O. Personal communication, 1957.

Dr. Dillingham suggested that if phenylalanine and tyrosine are taken from the cell protein to form lignin, the depletion of the protein may be the cause of the cessation of growth in the cells.

63. Pearl, I. A., Beyer, D. L., Johnson, B., and Wilkinson, S., Tappi 40, no. 7:374-8(July, 1957).

Alkaline hydrolysis of Populus tremuloides yielded syringaldehyde and vanillin in a ratio of 3:2. The same treatment produced p-hydroxybenzoic acid from all trees of the Populus genus and the closely related Salix genus but from none of the other 26 species of trees tested.

64. Freudenberg, K., Ann. Rev. Biochem. 8:81-112(1939).

Freudenberg states that lignin exists in different condensation steps according to the age and origin of the wood.

65. Pearl, I. A., and Beyer, D., Tappi 40, no. 1:45-54(Jan., 1957).

Approximately one-third of the methoxyl groups of aspenwood were found to be associated with something other than the Klason lignin or extractives. It is suggested that true aspen lignin may be present in aspenwood to the extent of 24-5% with an over-all methoxyl content of

15-16%. Para-hydroxybenzoic acid was identified as a major component of the extractives of P. tremuloides.

66. Nord, F. F., and Schubert, W. J., Tappi 40, no. 4:285-94(April, 1957).

The infrared spectra of Brauns' lignin of maple, oak, birch, and white Scots pine are presented.

67. Lyness, W. I., and Schenker, C., Tappi 40, no. 10:791-4(Oct., 1957).

The infrared spectra of Brauns' lignin from black spruce and slash pine are presented.

68. Smith, D. C. C., J. Chem. Soc. 1955:2347-51.

Brauns' lignin was isolated from Populus tremula. Alkaline hydrolysis of the lignin yielded p-hydroxybenzoic acid. The rate of the hydrolysis was taken as an indication that the acid was combined to the lignin by ester linkages between the carboxyl group of the acid and hydroxyls of the lignin. Esterification of the phenolic hydroxyl group of the acid or self-esterification were ruled out.

69. Kremers, R. E. Personal communication, 1957.

Salicin has been isolated from extracts of soft xylem from Populus tremuloides.

70. Feigl, T. Spot Tests. 4th ed. p. 226. New York, Elsevier Publishing Co., 1954.

Epihydrin which is characteristic of the aldehydes present in rancid fats and oils can be readily detected by its red color with phloroglucinol in hydrochloric acid.

71. Prager, B., Jacobson, P., Schmidt, P., and Stern, D. Beilstein's Handbuch der Organischen chemie. 4th ed. Vol. 8. p. 257. Berlin, Springer-Verlag, 1925.

The properties of the 2,4-dinitrophenyl ether of vanillin are described.

72. Blatt, A. H., editor. Organic syntheses. Collective Vol. 2. New York, John Wiley and Sons Inc., 1943. 126 p.

Directions for preparation of chelidonic acid are given. Ethyl oxalate, sodium ethoxide, and acetone are reacted together, then treated with cold hydrochloric acid to produce sodium chelidonate. Hydrolysis in hot hydrochloric acid yields hydrated chelidonic acid which is then dried to remove water of crystallization.

73. Willstätter, R., and Pummerer, R. Chem. Ber. 37:3740-52(1904).

The preparation of gamma pyrone by the distillation at 97°C., 13 mm. pressure of a mixture of chelidonic acid and copper powder is described. The distillate is dissolved in ether containing a small quantity of sodium carbonate to neutralize any chelidonic acid carried over. Redistillation of the solids gives gamma pyrone which crystallizes as it condenses. The compound can be recrystallized from hot petroleum ether or carbon disulfide.

74. Ross, A., Proc. Roy. Soc. (London) 113, no. A763:208(1926).

The infrared spectrum of a liquid film of gamma pyrone is given from the range of 1-10 mu. Strong absorption bonds at 3.0, 7.6, and 8.4 mu. indicate that the sample was contaminated with water.

75. Kremers, R. E. Personal communication, 1957.

Dr. Kremers stated that during the growing season freshly cut aspen logs from which the bark has been peeled take on a reddish color. When the newly formed outer tissue is removed and placed in methanol, the color disappears.

76. Lindstedt, G., Acta Chem. Scand. 4:448-55(1950).

A spray reagent for phenolic materials on paper chromatograms was prepared by suspending 5 g. of benzidene in 14 ml. of concentrated hydrochloric acid and dissolving the suspension in 980 ml. of water. Before using, this solution was mixed (1:1) with 10% sodium nitrite. It must be sprayed within 10 minutes. Dissolving a small amount of sodium carbonate in the mixture before using aids in color development.

APPENDIX I

PREPARATION OF A NEW DERIVATIVE OF SYRINGALDEHYDE

The 2,4-dinitrophenyl ether derivatives of two guaiacyl compounds, vanillin and dehydrodiconiferyl alcohol (71, 25) have been reported, but no syringyl derivatives are mentioned in the literature. Therefore, before attempting to prepare the derivative of sinapaldehyde (cf. Identification of Sinapaldehyde) the more readily available syringaldehyde was used first.

The 2,4-dinitrophenyl ether of syringaldehyde was prepared by dissolving 0.24 g. of syringaldehyde in 10 ml. of acetone and adding 0.24 g. of 1-fluoro, 2,4-dinitrobenzene, and 0.07 g. of sodium bicarbonate dissolved in 4 ml. of water. White crystals formed when the mixture was cooled. These were recrystallized from hot acetone. The melting point of the recrystallized material was 170-4°C.

APPENDIX II

SYNTHESIS OF GAMMA PYRONE

Because of misleading data obtained from the ultraviolet and infrared spectra of a contaminated eluate of sinapaldehyde, it was thought that gamma pyrone might be one of the ether-soluble materials. To check this, chelidonic acid was synthesized (m.p. 257°C.)(72), and gamma pyrone obtained by dry distillation of the chelidonic acid (73). The low melting point of gamma pyrone (32.5°C.) and its very hygroscopic nature prevented an accurate melting point determination; therefore, its identity was verified by preparation of its picrate (m.p. 125°C.).

Figure 10 shows the infrared spectrum of anhydrous gamma pyrone. Previously only a portion of the spectrum of the compound has been reported, and there was evidence that the sample used for that determination was contaminated with water (74).

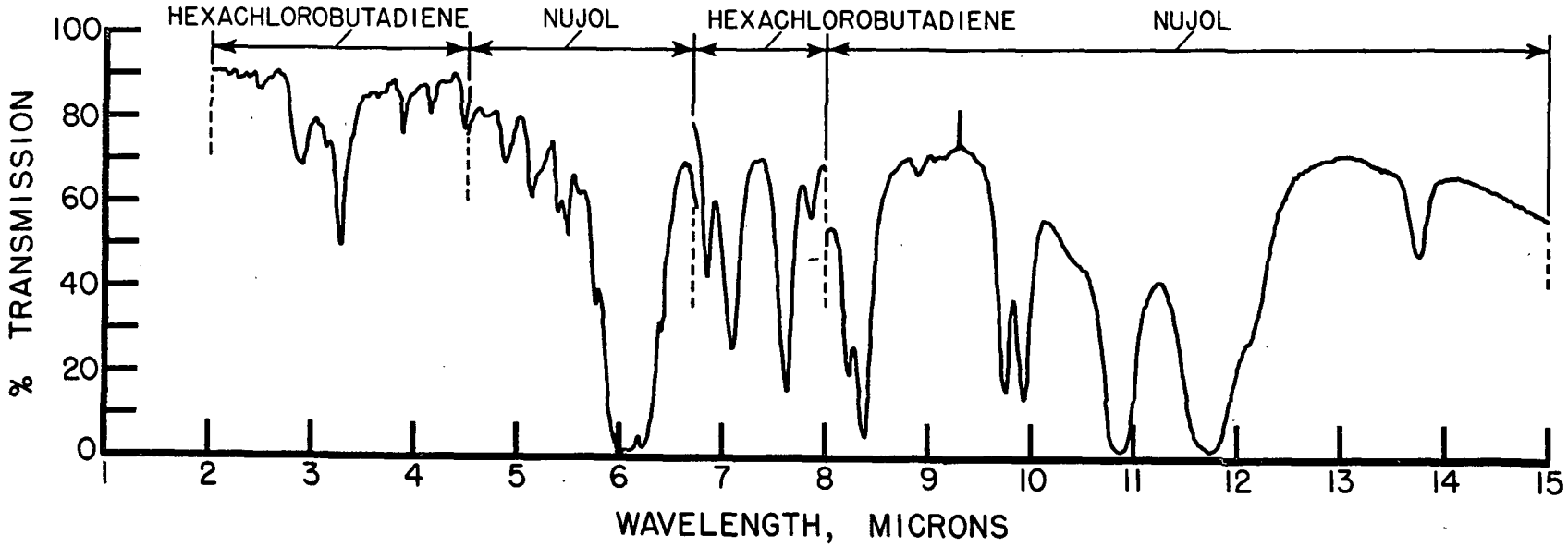

Figure 10. Infrared Spectrum of Gamma Pyrone

APPENDIX III

A RED PIGMENT

In one of the preliminary experiments, the water-soluble fraction, prepared from an extract of aspen new xylem collected in 1955 for another project, was extracted with benzene. The benzene extract was chromatographed on paper using the TAW developer, and the spot corresponding to sinapaldehyde was eluted with methanol. When the methanol solution was concentrated, it went from colorless, to yellow, to orange and finally to blood-red when all the methanol had been removed. When redissolved in a small amount of methanol, the color disappeared although it was regained when the solution was evaporated again. This phenomenon was repeated when benzene was used as the solvent; however, the red material could not be dissolved in ether. No further study was made of this material and no similar material was found in other extracts.

This pigment was considered to be of interest since a red coloration has been observed to form on the surface of freshly barked aspen logs (75). The color disappears when the outer tissue (soft xylem) is removed from the logs and placed in methanol.