THE CHEMICAL COMPOSITION OF THE EXTRACTIVES FROM THE NEWLY FORMED INNER BARK OF DOUGLAS FIR, *PSEUDOTSUGA MENZIESII* (MIRB.) FRANCO

by

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INTRODUCTION

In recent years a considerable amount of interest has been centered on the nature of the phenolic materials of conifer barks, particularly those from Douglas fir. It has been felt that a better understanding of these substances may lead to a more efficient use of the bark.

A major portion of the Douglas fir bark available from lumber and pulp mills is currently burned as fuel. However, uses have been found for several portions of the Douglas fir bark. Dihydroquercetin and quercetin, extracted from the bark, have limited use in the pharmaceutical industry, and they also exhibit highly desirable antioxidant properties.

The wax extracted from Douglas fir bark is also potentially valuable; while the tannins are used in the leather tanning industry. The major portion of the bark, which is not extractable with inert solvents, is separated by physical means into various fractions, some of which are used as plastic extenders.

One of the most interesting, and least understood, fractions of conifer barks is the bark lignin, or phenolic acid. This substance, which as the name implies contains both carboxylic and phenolic groups, can be removed from

extractive-free bark by extraction with a dilute sodium hydroxide solution. It can be obtained in yields of greater than fifty per cent from some parts of the bark (40). This material resembles wood lignin in many respects. However, the phenolic acid contains fewer methoxyl groups than lignin, and lignin is not believed to contain carboxyl groups (7).

Due to the severe conditions of isolation, the existence of carboxyl groups before isolation may be doubted. However, it is known that wood lignins do not develop carboxyl groups with alkali under similar conditions (33). Thus, it is obvious that the phenolic acids differ from wood lignin considerably.

Degradative oxidation of the phenolic acid by alkaline nitrobenzene has been shown to yield small amounts of vanillin and protocatechualdehyde with traces of syringaldehyde and p-hydroxybenzaldehyde (40). Softwood lignins yield predominantly vanillin and in much higher yields.

The phenolic acid from white fir, which has been shown to be very similar to that from Douglas fir, has been degraded by several different methods (13). Alkaline fusion gave relatively high yields of protocatechuic and oxalic acids with significant amounts of catechol and phloroglucinol. It is interesting to note that phloroglucinol has never been detected in the degradation products of wood

lignin (7). This suggests a marked difference between wood lignin and bark phenolic acid.

Another bark lignin fraction can be extracted by means of dioxane-hydrochloric acid (40). This substance resembles wood lignin more closely in that it has a similar methoxyl content (up to 15 per cent). However, this material does not give a positive Wiesner's test as does wood lignin, thus indicating a structural difference. The Wiesner test consists of treating the material with phloroglucinol and hydrochloric acid. A reddish color is produced by substances containing a free coniferylaldehyde structure (48).

Since most of the previous work on bark lignin has been confined to studies of their degradation products, it was felt that a different approach might be desirable. It seemed that an examination of the extractives of the newly formed inner bark might reveal the existence of low molecular weight precursors of the bark lignins. It is generally believed that minerals, water and reserve food flow up through the sapwood of trees to the leaves where photosynthesis occurs. The photosynthetic products then flow down the inner bark and are made available to the cambium where wood and bark synthesis occur. Thus, it is evident that any compounds present in the newly formed inner bark may be precursors for wood, bark, both or neither. However, it was believed that knowing the chemical composition of

the newly formed inner bark may contribute to the elucidation of the structure of the bark lignin.

The extractives of the newly formed xylem from aspen have been examined and were shown to contain several proposed precursors of wood lignin (43, 36). The extractives of the inner bark of aspen have also been characterized (12). Ten aromatic compounds were identified. They were: pyrocatechol, benzoic acid, vanillic acid, p-hydroxybenzoic acid, p-coumaric acid, salireposide, salicin, populin, tremuloidin and ferulic acid. Several of these compounds have been postulated as intermediates in the biochemical synthesis of wood lignin.

Recently Hergert (24) indicated the presence in Douglas fir bark of several monomeric phenolic substances. They were: protocatechuic acid, phloroglucinol, ferulic acid, coniferaldehyde and vanillin. It is interesting to note that each of these types of aromatic nuclei has been found in the degradation products of bark lignin.

The object of this research was to identify some of the relatively simple compounds in the newly formed inner bark extract of Douglas fir. As stated previously these substances may be precursors of wood, bark, both or neither. Therefore, a comparison of the extractives from the mature inner bark and outer bark with those from the newly formed inner bark was thought desirable. However, a much more thorough examination was made of the newly formed inner bark.

EXPERIMENTAL

COLLECTION OF SAMPLES

All of the samples used in this work were collected about ten miles west of Corvallis, Oregon in the Woods Creek area. The outer bark was removed with a hatchet and then the inner bark was peeled from the tree by hand. Immediately after removal from the trees the white, soft tissue from the cambial area of the bark was scraped from the inner bark with a knife. The moist, translucent, newly formed inner bark was immediately immersed in absolute methanol to stop enzyme activity. The remainder of the inner bark was also placed in methanol with the exception of those samples which were intentionally allowed to air-dry in order to determine the effect of this treatment upon the chemical composition.

PRELIMINARY INVESTIGATION

Effect of trees' age. In order to evaluate the effect of the age of the trees upon the composition and amount of the extractives the following samples were collected in June 1959. One sample was taken from a Douglas fir estimated to be about 200 years old which was approximately 4 feet in diameter at the base. The other sample was a combined sample from three second-growth Douglas fir trees, each about forty years old and one foot in diameter at the base.

Only the newly formed inner bark was collected from each tree.

After 24 hours the methanol extracts were decanted and each sample was covered with 90 per cent methanol and refluxed for one hour. The extracts were decanted, and the extraction with boiling, 90 per cent methanol was repeated one more time. The three extracts from each sample were combined, and the combined extracts were filtered and then evaporated under vacuum at 35° C. to remove all of the methanol.

The aqueous solution of the two extracts from each of the newly formed inner bark samples was then fractionated by a procedure similar to that used by Sultze (43) and Mugg (36). After thorough extraction with hexane the extracts were filtered through Celite filter beds in a Buchner funnel. The Celite mat was air dried and then extracted in a Soxhlet extractor with methanol.

The clear aqueous filtrate from the Celite filtration was then extracted with ethyl ether in a liquid-liquid extractor for 72 hours. The ether extract was evaporated to a convenient volume and extracted exhaustively in a separatory funnel with saturated sodium bisulfite, 5 per cent sodium bicarbonate and 5 per cent sodium hydroxide solutions successively.

The three resulting aqueous extracts were acidified with dilute hydrochloric acid and then exhaustively extracted

with ethyl ether. The above procedure resulted in seven fractions for each sample, i.e., hexane solubles, methanol solubles, water solubles and ethyl ether soluble aldehydes, acids, phenols and neutrals. This separation scheme is summarized in Figure 1.

The yield of each organic solvent soluble fraction was determined by evaporating the solution in an air stream and then drying overnight *in vacuo* at 55° C. The yield of the water soluble extracts was determined by evaporating an aliquot on a steam bath and drying to a constant weight at 105° C. The percentage yields were calculated by assuming that the sum of the recovered extractives and the marc equalled the total weight of the unextracted, moisture-free bark. The yields are given in Table 1.

Table 1

YIELD OF EXTRACTIVES FROM DOUGLAS FIR NEWLY FORMED INNER BARK

(Percentages based on moisture-free, unextracted bark)

				Ethyl ether soluble			
Sample	Hexane Solubles	Water Solubles	Methanol Solubles	Aldehydes	Acids	Phenols	Neutrals
40 year old trees	1.21	31.2	0.75	0.12	0.04	0.40	0.36
200 year old trees	0.99	40.3	0.59	0.09	0.02	0.01	0.01

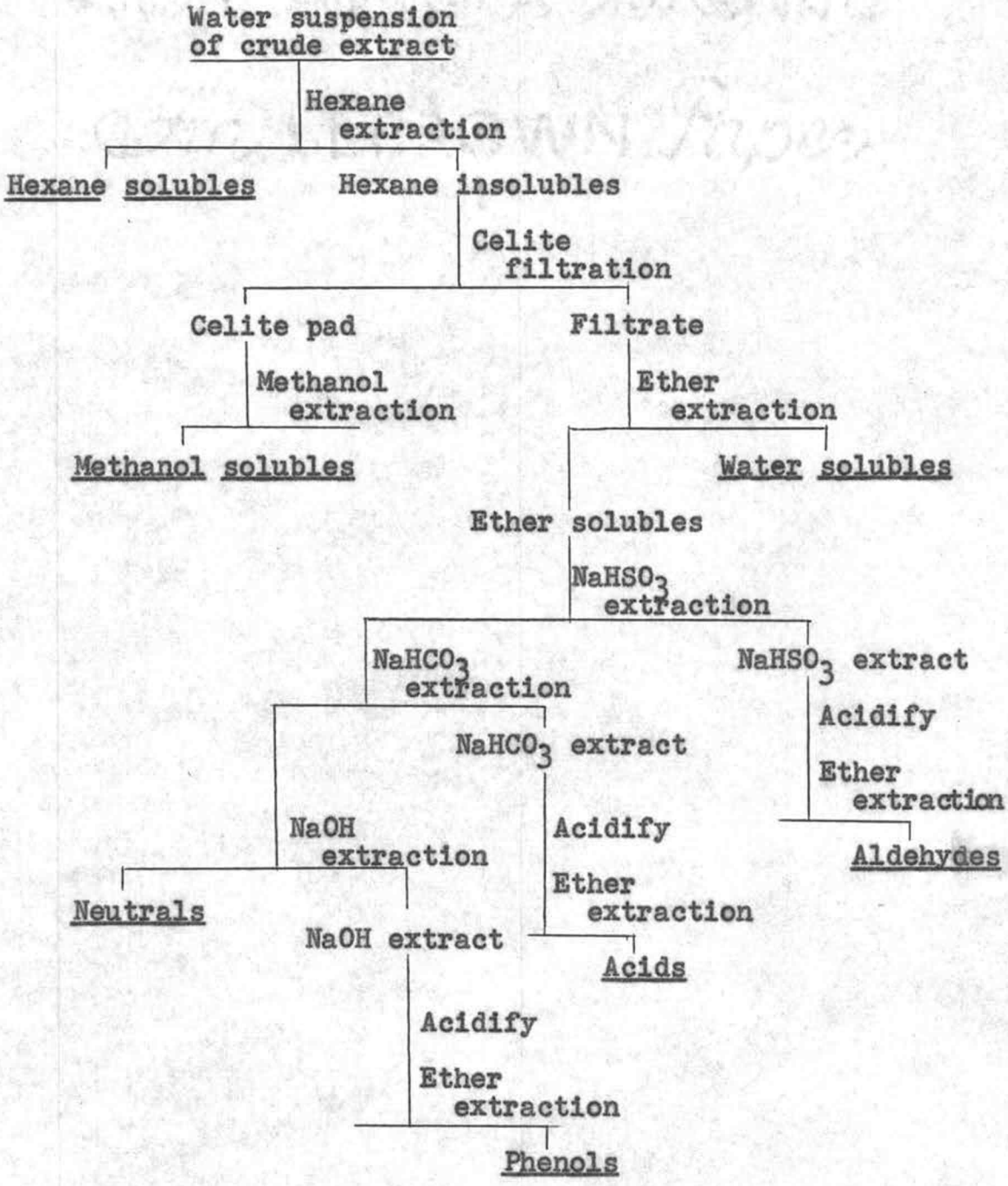


Figure 1. SEPARATION OF CRUDE BARK EXTRACT

Using these data the water soluble fractions from each sample were diluted to three per cent solids content for further work.

Water solubles. In an attempt to isolate amino acids, fifty ml. of the water soluble fraction were acidified to pH 2 with dilute hydrochloric acid and was added to a 1.5 x 60 cm. column of Dowex 50 X-4 cation exchange resin. The column was then washed with distilled water until the effluent gave negative tests to both the Molisch test for carbohydrates and the ferric chloride test for phenols.

The column was then eluted with two normal ammonium hydroxide. The eluent was dark red and gave a positive test for phenols. The dark color made a ninhydrin test for amino acids difficult to evaluate. A similar experiment was performed, but the column containing the sorbed material was washed thoroughly with methanol before elution with ammonium hydroxide. This time the amount of phenolic material was less as indicated by the color of the effluent. However, it was evident that a large amount of phenolic material was still present.

Thus, it appeared that ion exchange methods were not suitable for the isolation of amino acids from the water soluble fraction without previous removal of the phenolic material.

The water soluble fraction was next examined paper chromatographically for carbohydrates. The chromatograms

were developed descendingly on Whatman 1 paper using BAW = (n-butanol-acetic acid-water (4:1:5)). The developed chromatograms were sprayed with p-anisidine hydrochloride (6). Sucrose, glucose and fructose appeared to be present in both the fraction from the 200 year old tree and the 40 year old trees in approximately equal concentrations.

When a chromatogram developed as above was sprayed with 0.2 per cent ninhydrin in n-butanol saturated with water (6) several very weak amino acid spots appeared in both fractions.

Ether solubles. The ether soluble aldehydes, phenols, acids and neutrals were chromatographed using the BAW developer. The chromatograms were examined with ultraviolet light, dipped in Barton's phenol indicator (4), and sprayed with two per cent 2,4-dinitrophenylhydrazine in two normal hydrochloric acid (5) and diazotized p-nitroaniline (6). These indicators showed that all of the fractions were essentially identical. The main constituents of each fraction caused an elongated spot from Rf 0.5 to 0.7 which appeared to be composed of two partially merged spots. This spot was dark blue in ultraviolet light, dark blue when dipped in Barton's reagent and bright orange with diazotized p-nitroaniline. No color was observed with 2,4-dinitrophenylhydrazine.

When the ether soluble fractions were chromatographed using two per cent acetic acid as the developing solvent

a similar elongated spot appeared between Rf 0.3 and 0.5. Thus, it was evident that a classical separation into aldehydes, acids, phenols and neutrals was to no avail with these extracts.

Comparison of bark fractions. Although the above cursory examination demonstrated that the extractives from the two different aged trees were qualitatively similar, the second-growth trees contained more ethyl ether soluble materials. It was also possible to collect a more random sample from second-growth trees due to the much larger number of these trees being available. Therefore, the next sample was collected in September 1959 from ten trees estimated to be about forty years old, each between one and two feet in diameter at the base.

As described previously the outer bark was removed with a hatchet, but this time a portion of the outer bark and the mature inner bark were immediately placed in methanol. The newly formed inner bark was collected as described previously, and it was also placed in methanol.

The samples were extracted with two changes of boiling 90 per cent methanol for one hour each. These extracts were added to the decanted absolute methanol extracts. The solution was filtered to remove fibrous material, and the methanol was removed from the combined fractions under vacuum at 35° C.

The aqueous solutions were then extracted exhaustively with hexane in a separatory funnel and then filtered through a Celite filter bed. The Celite, after air-drying, was extracted with methanol in a Soxhlet extractor.

The filtered aqueous solution was then exhaustively extracted with ethyl ether in a liquid-liquid extractor. This separation scheme is illustrated in Figure 2. No attempt was made to separate the ethyl ether solubles into aldehydes, acids, phenols and neutrals since previous work had shown that essentially no separation occurred.

The yield of each fraction was determined as described previously and is reported in Table 2.

Table 2

YIELD OF EXTRACTIVES FROM DOUGLAS FIR BARK

(Percentages based on moisture-free, unextracted bark)

Sample	Methanol Solubles	Hexane Solubles	Water Solubles	Ethyl ether Solubles
newly-formed inner bark	1.13	2.20	39.8	0.69
mature inner bark	1.71	1.02	18.4	0.42
outer bark	3.66	2.38	19.1	1.21

When the extract from the outer bark was filtered, before removal of the methanol, 1.07 grams of a tan wax was collected on the filter paper. It was dissolved in ethyl ether, filtered and dried *in vacuo* at 55° C. This material

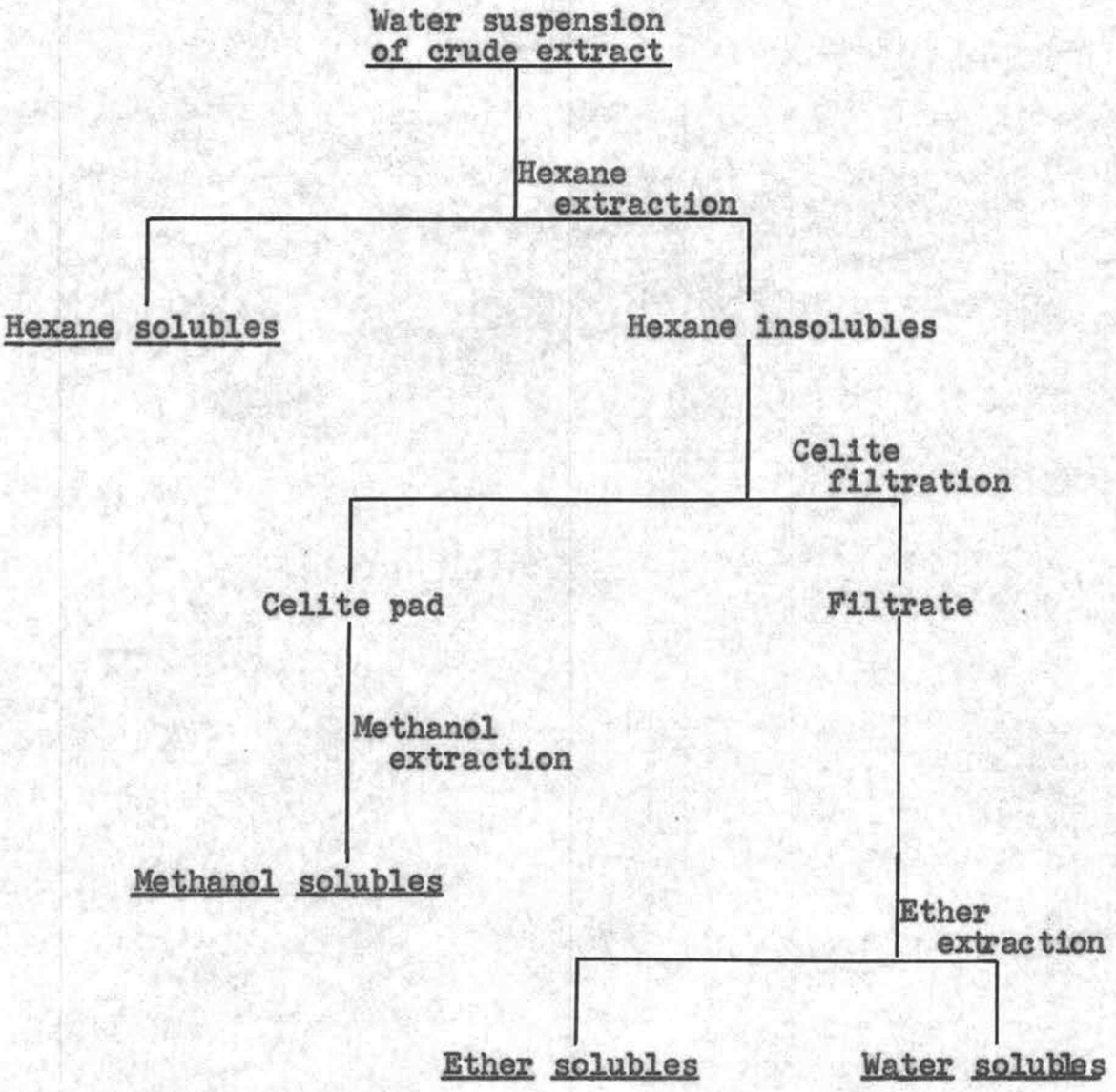


Figure 2. SEPARATION OF CRUDE BARK EXTRACT

was soluble in hexane and was added to the hexane solubles from the outer bark and is reported as such in Table 2.

Hexane solubles. The hexane soluble extractives from the newly-formed inner bark was a dark brown, sticky syrup. This fraction from the inner bark was also dark-brown and sticky but much more viscous. The outer bark hexane soluble fraction was a brown, slightly sticky wax. No further work was done with these fractions.

Water solubles. In order to prevent decomposition of the water soluble fractions they were covered with a layer of toluene and were kept in the refrigerator. It was decided to determine which sugars were present in the three water soluble fractions by paper chromatography. The developing solvent chosen was n-butyl acetate-pyridine-ethanol-water (8:2:2:1) (19).

Sugars. Sugars have very low Rf's in this solvent, but by running the solvent off the edge of the paper excellent separation occurs. Since the p-anisidine hydrochloride reagent is not very sensitive to sucrose and fructose, another spray reagent, 1 per cent alcoholic α-naphthol-phosphoric acid (10:1) (6), was used for these two sugars.

Of the seven known sugars run in parallel with the unknowns only mannose and raffinose were not found in any of the bark fractions. These results are given in Table 3.

Table 3

SUGARS FOUND BY PAPER CHROMATOGRAPHY IN DOUGLAS FIR BARK EXTRACTIVES

Sugar	Newly formed inner bark	Mature inner bark	Outer bark
arabinose	-	-	trace
glucose	trace	+	+
mannose	-	-	-
xylose	-	trace	trace
sucrose	+++	++	+
fructose	trace	++	+
raffinose	-	-	-

Since each fraction contained glucose, fructose, and sucrose in considerable quantities it was decided to make quantitative estimations of these sugars. Exactly two lambdas of the extracts were placed on chromatograms along with various known concentrations of glucose, fructose and sucrose.

After development duplicate chromatograms were sprayed with p-anisidine hydrochloride to indicate glucose and α-naphthol-phosphoric acid to indicate sucrose and fructose. The quantity of each sugar was then estimated by visual comparison with the standard sugars. These results are found in Table 4.

Table 4

ESTIMATED QUANTITIES OF SUGARS IN DOUGLAS FIR BARK EXTRACTIVES

(Percentages based on moisture-free, unextracted bark)

Sample	Sucrose	Glucose	Fructose	Total
newly-formed inner bark	16.0	trace	trace	16.0
mature inner bark	2.6	1.3	3.9	7.8
outer bark	0.6	0.6	0.6	1.8

It is interesting to note that the sugars constituted approximately 40 per cent of the newly-formed inner bark and mature inner bark water solubles. However, less than 10 per cent of the outer bark water solubles were sugars.

Phenols. An investigation was next made of the phenolic materials in the water soluble fractions, since it would seem that the most direct evidence of bark lignin synthesis might be obtained by comparing the phenolic constituents of the three samples.

Chromatograms were prepared of the water soluble fractions using water saturated with carbon dioxide as the developing solvent. This solvent has been found to be quite successful in separating complex phenolic mixtures (16).

The chromatograms were sprayed with diazotized p-nitroaniline, followed by an overspray with 20 per cent

aqueous sodium carbonate. The three fractions gave similar chromatograms. A tan streak extended from the origin to an Rf of about 0.6, while a faint gray-violet streak extended from Rf 0.6 to 0.9. Thus, it appeared that the phenolic constituents must be fractionated by some other means before paper chromatography could be used to show differences in the three fractions.

Recently Gardner and co-workers (16) isolated a phenolic acid compound from Western cedar. They precipitated the phenolic material from an aqueous extract with lead acetate. The lead-phenol complex was decomposed with acidified methyl ethyl ketone which also dissolved the phenolic material. After removal of the neutral and weakly acidic substances, evaporation of the dried methyl ethyl ketone solution gave chromatographically pure "plicatic acid". Its structure was not completely elucidated, however it was shown to contain guaiacyl and carboxyl groups and had a molecular weight of approximately 433. This compound was obtained in a 36 per cent yield, based on the lead-phenol complex.

When this method was applied to the water extracts from the three bark fractions, only very small amounts of tannin-like substances were recovered. It was found that drying the methyl ethyl ketone caused most of the colored material to precipitate.

An attempt was also made to separate the phenolic substances by extraction of the water solutions with ethyl acetate. It was found that it was necessary to saturate the aqueous layer with sodium chloride in order to obtain any substantial amount of phenolic material in the ethyl acetate layer. The saturation of the aqueous layer with sodium chloride caused most of the coloring matter to precipitate. This precipitate was not soluble in the ethyl acetate but was soluble in distilled water, and appeared to be tannin.

The ethyl acetate layers were filtered, dried over magnesium sulfate and evaporated to dryness in a rotary evaporator to give orange solids.

The ethyl acetate solubles were chromatographed using two per cent acetic acid, BAW, distilled water saturated with carbon dioxide and secondary butanol saturated with water. Indicators used were: Barton's reagent, diazotized p-nitro aniline, cinnamaldehyde-hydrochloric acid, p-toluene sulfonic acid, and five per cent hydrochloric acid in n-propanol. The first two are general phenol indicators, while the latter two are indicators for leucoanthocyanidins (24). Cinnamaldehyde-hydrochloric acid seems to be specific for unconjugated phloroglucinol or resorcinol derivatives and pyrogallol compounds (24). It is generally used to indicate substances with flavan-type structures.

Each of the solvents produced streaks with each of the fractions irrespective of the indicator used. It was noted that the streaks were denser in certain areas, but these areas corresponded to materials later found in the ethyl ether soluble fractions, thus indicating incomplete extractions with ethyl ether.

Infrared spectra of these fractions were determined using the potassium bromide pellet method. The three curves were identical except that the spectrum of the outer bark extractives exhibited a weak carbonyl absorption band at 1690 $cm.^{-1}$. This band could be accounted for by the presence of a small amount of d-dihydroquercetin in this fraction. However, the carbonyl band of d-dihydroquercetin occurs at 1642 $cm.^{-1}$ (26). No explanation could be given for this discrepancy.

Several small scale counter-current extractions were made using ethyl acetate and methyl ethyl ketone as the organic layers and distilled water as the non-organic layer. However, no separation was evident using paper chromatography as an indicator.

Phenolic substances in aqueous solutions have also been separated by sorbing on cation exchange resins followed by elution with ethanol (14). No separation appeared to result when this method was used.

Ether solubles. The ethyl ether solubles were light brown solids and were soluble in acetone, ethanol and hot

water. Their aqueous solutions became green when ferric chloride was added, indicating the presence of catechol-like structures. A portion of each sample was dissolved in acetone to make a one per cent solution suitable for paper chromatography. The initial chromatograms were developed with two per cent acetic acid. With Barton's phenol indicator two spots appeared from the newly formed and mature inner bark. The outer bark gave only one spot. When run in parallel with authentic d-dihydroquercetin, all three fractions exhibited a spot with an Rf on 0.35, which was identical to the Rf of d-dihydroquercetin. The other spot on the chromatograms of the two inner bark fractions had an Rf of 0.45.

When BAW was used as the developing solvent, it was found that the newly formed inner bark exhibited spots at Rf 0.67 and 0.56, the inner bark showed the same two spots with a very faint spot at Rf 0.79 and the outer bark showed a spot at Rf 0.79. It appeared that the principal constituents of the ethyl ether soluble materials from the inner bark fractions were not d-dihydroquercetin, since d-dihydroquercetin was found at Rf 0.79.

When duplicate chromatograms were sprayed with cinnamaldehyde-hydrochloric acid only the two spots in the inner bark fractions became visible. This suggested that one of the spots may have been due to d-catechin, since this compound responds to this spray reagent and has a

similar Rf. Chromatograms prepared with the mature inner bark and newly-formed inner bark ether solubles run parallel to an authentic sample of d-catechin further substantiated this possibility.

d-Catechin. From the above chromatography experiments it was decided to attempt a crystallization of the ether solubles from the three fractions. A small amount of the newly formed inner bark ether solubles was dissolved in ethanol, chloroform was added until the solution became slightly cloudy, and the solution was placed in the refrigerator. After several days a small amount of a light colored material had precipitated. This was removed by filtration, air-dried and a melting point determination was made. It appeared to undergo a phase change at 146 to 149° C. and melted at about 175° C.

A portion of the ether soluble fraction from the mature inner bark was dissolved in a small amount of hot water, treated with a small amount of Norite and filtered. After several days in the refrigerator a light tan precipitate had formed. This was removed by filtration, dried in vacuo at 55° C., and its melting point characteristics were the same as those of the material isolated from the newly formed inner bark by precipitation from ethanol-chloroform. A mixed melting point of the two fractions was not depressed. Therefore, the two fractions were combined.

When the combined solids were recrystallized from hot water and dried, the material sintered at 150° C. and melted at 176 to 177° C. These melting point characteristics are identical to those given in the literature (26) for d-catechin. A mixed melting point with authentic d-catechin was not depressed.

The infrared spectrum of this substance was obtained by the potassium bromide pellet method using a Perkin-Elmer model 21 infrared spectrophotometer. This spectrum was identical to a spectrum of an authentic sample of d-catechin determined concurrently. The absorption bands were identical to those found by Hergert (26).

d-Dihydroquercetin. A portion of the ether soluble fraction from the outer bark was dissolved in hot water and treated with a small amount of Norite. After several days in the refrigerator nearly white crystals had formed. These were collected by filtration and recrystallized twice from hot water. The colorless crystals, after drying *in vacuo* at 55° C., melted at 246 to 248° C., undepressed when mixed with authentic d-dihydroquercetin. The melting point of d-dihydroquercetin is given in the literature as 241 to 243° C. (26). By fractional crystallization d-dihydroquercetin can be obtained with a melting point of over 250° C.

Thirty mg. of this material were dissolved in 3 ml. of acetic anhydride and 0.45 ml. of pyridine. After

standing for 24 hours the solution was poured into cold water, and the resulting precipitate was recrystallized twice from methanol. The acetate melted at 129 to 130° C. The acetate of authentic d-dihydroquercetin prepared in the same manner melted at the same temperature. The melting point of the acetate is given as 128 to 129° C. (26).

l-Epicatechin. Several attempts were made to crystallize the material responsible for the other spot on the inner bark chromatograms; however, these attempts were unsuccessful. The intensity of the spot indicated that this compound was probably present in somewhat lesser amounts than the d-catechin.

A search of the literature (25) revealed that the Rf's of this substance in two per cent acetic acid and BAW were similar to those reported for l-epicatechin.

Attempts were made to prepare an infrared spectrum of this substance. The ether soluble fraction from the newly formed inner bark was streaked on eight 9 inch strips of Whatman 3MM chromatography paper and the chromatograms were developed with BAW. Guide strips were cut from the edges and dipped in Barton's reagent. The bands formed by this compound, although very near, appeared to be separated from the bands corresponding to d-catechin. The bands were cut from the chromatograms and eluted with methanol in a micro-Soxhlet extractor.

The methanol was filtered and evaporated to less than one ml., and 250 mg. of powdered potassium bromide were added. The methanol was removed under vacuum, and the powder was dried *in vacuo* at 55° C. over-night. The infrared spectrum of a pellet prepared from this powder was determined and compared with the spectrum of an authentic sample of l-epicatechin treated in the same manner. The authentic l-epicatechin was kindly supplied by Dr. Simon Wender of the University of Oklahoma. The absorption bands of the authentic l-epicatechin were very sharp and were identical to those found by Hergert (26). The bands of the inner bark ether soluble material were broad, and many of the close bands were not resolved. It appeared that the unknown material was an impure sample of l-epicatechin.

Attempts were made to isolate a moderate amount of this material by a modification of a method described for the separation of up to three grams of a mixture of amino acids by chromatography on seed-test paper (10). However, none of the solvents used separated this substance sufficiently from the d-catechin.

Chromatography series. In order to further substantiate the presence of the three flavonoid compounds, a series of chromatograms were prepared using six different developing solvents. Each chromatogram was prepared by adding spots of authentic d-catechin, l-epicatechin and

Table 5

RF'S OF d-CATECHIN, l-EPICATECHIN AND d-DIHYDROQUERCETIN IN VARIOUS SOLVENTS

Solvent	Rf x 100 C[a]	E[a]	DHQ[a]	nfib[a]	mib[a]	ob[a]
BAW	70	59	85	59,70	59,70,(85)[b]	85,(92)[b]
2 % acetic acid	41	32	31	32,41	32,41	32,(52)[b]
n-butanol-pyridine-water (10:3:3)	90	87	96	89	89,(96)[b]	96
Ethyl acetate saturated with water	63	62	94	63	63,(94)[b]	94
60 % acetic acid	70	65	76	65,70	65,70,(76)[b]	76
60 % i-propanol	79	72	84	72,79	72,79,(85)[b]	85

a - C = d-catechin
E = l-epicatechin
DHQ = d-dihydroquercetin
nfib = newly formed inner bark ether sclubles
mib = mature inner bark ether solubles
ob = outer bark ether solubles

b - numbers in parentheses represent very faint spots

have been the degradation or polymerization of the catechins, brought about by air-drying.

In an effort to test this theory, 30 grams of inner bark were collected and allowed to air-dry for several months. At the end of this time the sample was ground in a Wiley mill to pass a 20 mesh screen and was exhaustively extracted with benzene to remove fats and waxes. The sample was air-dried and re-extracted with methanol. Two hundred ml. of water were added to the methanol solution and the methanol was removed under vacuum at room temperature.

The aqueous solution was then extracted with ethyl ether in a liquid-liquid extractor. The ether extract was evaporated to dryness in vacuo, and the residue was taken up in a small amount of acetone. Testing this solution with zinc and hydrochloric acid revealed that a substantial amount of d-dihydroquercetin was present.

The acetone solution was spotted on Whatman 1 chromatography paper, and the chromatograms were developed with BAW. Spots of the ether solubles extracted from an inner bark sample which was not allowed to air-dry were run in parallel, along with authentic d-catechin, l-epicatechin and d-dihydroquercetin. Triplicate chromatograms were treated with Barton's reagent, cinnamaldehyde-hydrochloric acid and zinc-hydrochloric acid. The zinc-hydrochloric acid reagent procedure consisted of dusting one side of

the chromatogram evenly with zinc powder and then spraying the other side with two normal hydrochloric acid. This reagent produced a red spot with d-dihydroquercetin but no noticeable color with d-catechin or l-epicatechin.

These chromatograms showed that although d-catechin and l-epicatechin were still present in the air-dried sample the relative amount of d-dihydroquercetin had increased greatly. It is obvious that this phenomenon may have been due to either an increase in the amount of d-dihydroquercetin or a decrease in the amount of the catechins. However, it was believed that this experiment established that prolonged air-drying of the bark could easily make isolation of d-catechin and l-epicatechin difficult due to the large excess of d-dihydroquercetin.

Analysis of marcs. Samples were collected of the newly formed inner bark, mature inner bark, and outer bark and allowed to air-dry. These samples were then ground in a Wiley mill to pass a 20 mesh screen, and 20 grams of each were extracted in a Soxhlet extractor with an azeotropic mixture of benzene and ethanol followed by 95 per cent ethanol. The samples were next extracted with three one liter portions of boiling water for one hour each. The samples were air-dried and placed in air-tight bottles. A small portion of each was used for a moisture determination. The TAPPI Standard 72 per cent sulfuric acid lignin determination (44) and the TAPPI

standard ash determination (44) were made of each sample. The results of these determinations are reported in Table 6.

Table 6

ANALYSIS OF EXTRACT-FREE DOUGLAS FIR BARK

(Percentages based on moisture-free, extracted bark)

Sample	Lignin	Ash
newly formed inner bark	18.79	5.19
mature inner bark	31.74	0.69
outer bark	79.35	0.21

The lignin determination results show that the "lignification" of bark is somewhat analogous to that of wood. However, in wood lignification the amount of lignin increases rapidly proceeding from the cambium, and the lignin content attains a constant value within a very short distance from the cambium.

The relatively high lignin content of the newly formed inner bark is quite unexpected. However, it has been shown that lignin determinations with sulfuric acid on bark do not give true representations of the amount of "lignin" present, especially in the cambial area, since much of the protein material is insolubilized by sulfuric acid and gives results which are far too high.

The exceedingly high ash content of the newly formed inner bark can be explained by the fact that a relatively

large amount of mineral matter is necessary in the cambial area in order to support the growing processes. These figures also show that the generally higher ash content of barks is not due to dust, etc., since the outer bark, which is completely exposed to such impurities, contains a relatively small amount of ash.

NEWLY FORMED INNER BARK

Another sample was collected in June 1960 consisting entirely of newly formed inner bark. This sample was to be used for a more intensive study of this fraction. The sample was collected from five trees, estimated to be about 40 years old, each between one and two feet in diameter at the base. The sample was placed in methanol immediately after removal from the trees.

The extraction and fractionation of the extract into hexane solubles, methanol solubles, ethyl ether solubles and water solubles was performed as previously described. The results of this fractionation are given in Table 7.

Table 7

YIELD OF EXTRACTIVES FROM NEWLY FORMED INNER BARK OF DOUGLAS FIR

(Percentages based on moisture-free, unextracted bark)

Fraction	Per cent
Hexane	1.57
Methanol	0.93
Ethyl ether	0.74
Water	42.0

Hexane solubles. In an attempt to characterize the hexane soluble fraction the following separation was made. A known amount of the hexane solubles was dissolved in ethyl ether which was then thoroughly extracted with a 5 per cent sodium bicarbonate solution. The bicarbonate solution was acidified and thoroughly extracted with ethyl ether. The ether was removed under vacuum to yield a free acid fraction.

The ether solution containing the neutral and weakly acidic substances was evaporated to dryness, and the residue was refluxed with a 10 per cent potassium hydroxide solution. The alkaline solution was cooled and extracted with ethyl ether, which upon evaporation gave an unsaponifiable fraction.

The alkaline solution was then acidified and extracted with ether to yield an ether soluble, saponifiable fraction. The aqueous solution contained a suspended solid

which was removed by filtration. This dark brown powder was insoluble in hexane, ethyl ether and water but soluble in acetone. The yield and description of each fraction are given in Table 8.

Table 8

FRACTIONATION OF HEXANE SOLUBLES FROM DOUGLAS FIR NEWLY FORMED INNER BARK

Fraction	% Yield	Description
Hexane solubles	100	Tan, sticky wax
Free acids	3	Dark brown wax
Unsaponifiables	5	Light brown wax
Saponifiables		
Ether solubles	66	Dark brown syrup
Acetone solubles	27	Dark brown amorphous powder

In another attempt to separate the hexane solubles a small portion of the hexane solubles was added to a 1.3 x 20 cm. column of alumina (activity grade II/III). The column was eluted with 100 ml. of each of the following solvents: petroleum ether, petroleum ether-benzene (9:1), petroleum ether-benzene (1:1), benzene, benzene-methanol (1:1) and methanol. The effluent was collected in 40 fractions of equal volume. Upon evaporation of the fractions in an air stream it appeared that very little separation had occurred. However, fractions 5 through 8 contained small needle-shaped crystals, melting point 130 to 132° C.,

which gave a positive Lieberman-Burchard test for sterols. Attempts to recrystallize this substance were unsuccessful due to its paucity.

Attempts were also made to isolate pure substances from the hexane solubles by fractional crystallization from hot acetone. An apparently colorless precipitate resulted which was collected by centrifugation. However, upon drying the material became a brown oil whose infrared spectrum had a strong carbonyl absorption band as its only outstanding feature. This brown oil could not be made to crystallize and was not further examined.

Methanol solubles. The methanol solubles were indistinguishable from the water solubles when examined paper chromatographically for phenolic substituents. It was noted that the methanol solubles contained a relatively high amount of ash. This fraction was not examined further.

Water solubles.

Sugars. A quantitative chromatographic study of the sugars in the water extract of this bark sample was made as described previously. It was interesting to note that, in contrast to the bark sample obtained in the fall, raffinose was found in relatively high concentrations in this sample. The quantitative results are given in Table 9.

Table 9

ESTIMATED QUANTITIES OF SUGARS IN DOUGLAS FIR NEWLY FORMED INNER BARK EXTRACT

(Percentages based on moisture-free, unextracted bark)

Sugar	%
Sucrose	27.0
Glucose	0.7
Fructose	0.7
Raffinose	0.5

Ion exchange separation. The water soluble fraction was next separated by ion exchange chromatography. Fifty ml. of the water solution containing 3.8 grams of solids were treated with saturated lead acetate solution to precipitate the polyphenolic substances. The suspension was filtered to remove the lead-phenol complex, and the filtrate was tested with lead acetate for complete precipitation of the phenolic materials.

The excess lead was then removed by passing hydrogen sulfide through the solution and filtering off the lead sulfide. The excess hydrogen sulfide was removed under vacuum.

The solution was then passed through a column of Dowex 50-x4 cation exchange resin in the hydrogen ion form. The bed volume was 25 ml., and the flow rate was adjusted to 25 ml./hr. The column was then washed with distilled water until a positive Molisch test for carbohydrates was not obtained.

The combined effluent was next passed through a column of Dowex 1-x4 anion exchange resin in the hydroxyl ion form. The bed volume was 25 ml., and the flow rate was 40 ml./hr. This column was also washed with distilled water until a positive Molisch test for carbohydrates was not obtained. The combined effluent was evaporated to about one ml., and was covered with a layer of toluene to prevent bacterial decomposition. It was designated "ib neutrals" and was stored in the refrigerator.

The Dowex 50 cation exchange column was eluted with two normal ammonium hydroxide until the effluent was distinctly basic to litmus. An additional 20 ml. of ammonium hydroxide were passed through the column followed by distilled water until the effluent was neutral. The combined effluent was evaporated in a rotary evaporator to yield 56 mg. of a red-brown powder. This fraction was designated "ib bases" and was placed in the refrigerator.

The Dowex 1 anion exchange column was eluted with two normal hydrochloric acid until the effluent was acid to litmus. An additional 20 ml. of hydrochloric acid were then added followed by distilled water until the effluent was neutral. The combined effluent was evaporated in a rotary evaporator to yield 94 mg. of a gray-violet, deliquescent solid. This fraction was designated "ib acids" and was also stored in the refrigerator.

Amino acids. The ib bases were examined paper chromatographically using BAW as the developer. After development the chromatograms were sprayed with 0.2 per cent ninhydrin in n-butanol saturated with water. Numerous spots were apparent, and therefore, the amino acids were estimated quantitatively by paper chromatography using n-butanol-acetic acid-water (25:6:25) and 80 per cent phenol (6). Known concentrations of 20 known amino acids were run in parallel. These results are shown in Table 10.

Table 10

AMINO ACIDS IN NEWLY FORMED INNER BARK OF DOUGLAS FIR

Amino acid	Approximate amount[a]
Aspartic acid	++
glutamic acid	++
serine	++
proline	+
glycine	+
alanine	+
threonine	+
valine	+
leucine	+
isoleucine	+
phenylalanine	trace (?)[b]
tyrosine	trace (?)[b]

a - ++ = greater than 0.01%, + = less than 0.01%; both based on moisture-free, unextracted bark
b - presence of these compounds doubtful

Hydroaromatic acids. The ib acids were next examined paper chromatographically. Since it was assumed that this fraction would contain uronic acids, a developer suitable for these compounds, i-propanol-pyridine-water-

acetic acid (8:8:4:1) (6), was chosen. After development it was found that spraying with aniline phthalate, naphthoresorcinol, or p-anisidine hydrochloride did not cause any spots to appear. Since each of these sprays should give colors with uronic acids (6), it was thought that the concentration of the acids might be quite low. However, even when large amounts of this fraction were placed on chromatograms no spots appeared with the three carbohydrate indicators.

Therefore, a series of indicators was used to locate other compounds on duplicate chromatograms. Only very faint traces of phenolic material were shown to be present, but an elongated spot appeared at Rf 0.45 with a bromphenol blue indicator (37) which is used to indicate the presence of strongly acidic materials. This same spot was colorless on a brown background when allowed to stand at room temperature over-night after spraying with 0.1 normal silver nitrate-5 normal ammonium hydroxide (1:1) (6).

The aqueous solution containing the compound(s) was diluted to 25 ml. and was extracted with three 25 ml. portions of ethyl ether. Spot tests of the layers, after evaporation to one ml. each, revealed that all of the strongly acidic materials remained in the aqueous layer, while the trace of phenolic material was transferred to the ether layer.

The aqueous layer was chromatographed on Whatman 1 paper using BAW. Spraying with the bromphenol indicator showed a strong spot at Rf 0.30. Silver nitrate-ammonium hydroxide showed the spot at Rf 0.30 strongly and a weak spot at Rf 0.12. Several other developing solvents gave little further evidence concerning the nature of these substances, although Rf's and one reagent, pyridine-acetic anhydride (37), did eliminate several likely aliphatic carboxylic acids, such as citric, lactic, oxalic, succinic, tartaric and glyceric acids. Also eliminated were the acids found in the Krebs tricarboxylic acid cycle.

A spot test of this fraction with Cartwright's hydroaromatic acid spray (11) gave a strongly positive reaction. This reagent does not give colors with sugars or polyphenols and is assumed to be specific for substances closely related to shikimic and quinic acids.

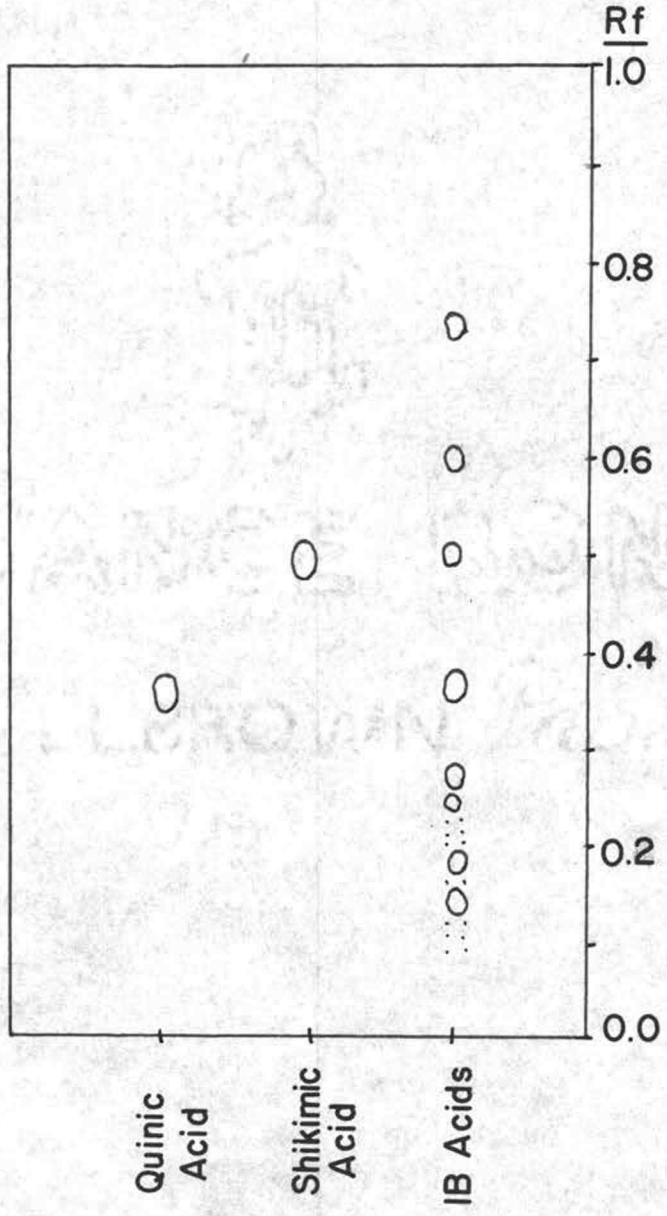
Chromatograms using ethyl acetate-acetic acid-water (3:1:3), gave strong yellow spots with this indicator at Rf's 0.37 and 0.51 with several other weak spots. A literature (15) search revealed that these Rf's correspond quite closely to those of quinic and shikimic acids, respectively. Therefore, authentic samples of these two acids were obtained, and chromatograms were prepared using ethyl acetate-acetic acid-water (3:1:3), n-butanol-pyridine-water (10:3:3) and phenol-water (3:1) with 0.9 per cent formic acid (22). In each of these solvents the two

strongest spots corresponded to quinic and shikimic acids. The ethyl acetate-acetic acid-water solvent gave the best resolution. A drawing of this chromatogram is shown in Figure 3.

Various concentrations of shikimic and quinic acids were used in preparing these chromatograms, and the approximate concentrations of the two acids in the extract were estimated. The concentration of shikimic acid was approximately 0.03 per cent, and that of quinic acid was 0.10 per cent, both based on the moisture-free, unextracted bark.

Since the hydroaromatic acids occur as phosphate esters in the biosynthesis pathway of bacteria, a duplicate chromatogram was sprayed with the phosphate indicator of Hanes and Isherwood (21). No spots were produced with this indicator, although a spot was found for phosphoric acid run in parallel.

Wiesner positive material. The ib neutrals were chromatographed using BAW as the developing solvent. The spots corresponding to glucose, fructose and sucrose were very strong since this solution was approximately 50 times as concentrated as the original water soluble fraction. Barton's indicator showed that this fraction contained a trace of phenolic material. A chromatogram sprayed with silver nitrate-ammonium hydroxide indicated a reducing substance at Rf 0.83 in BAW. It was also found that this spot



Developer: ethyl acetate-acetic acid-water (3:1:3)
Indicator: Cartwright's hydroaromatic reagent

Figure 3. CHROMATOGRAM OF HYDROAROMATIC ACIDS

appeared dark blue under ultraviolet light (no change with ammonia), gave no color with Barton's reagent or diazotized p-nitro aniline, but gave a strong orange color with Wiesner's reagent (48). Wiesner's reagent consists of one per cent phloroglucinol in 12 per cent hydrochloric acid and gives colors with lignin and other substances with the coniferyl group. When a chromatogram of this Wiesner positive material was sprayed with 2,4-dinitrophenylhydrazine a bright orange spot developed.

Chromatograms of this substance with authentic coniferaldehyde and vanillin using two per cent acetic acid and BAW as developing solvents indicated that it was neither of these compounds. These data are presented in Table 11.

Table 11

PAPER CHROMATOGRAPHY OF WIESNER POSITIVE MATERIALS

	Rf x 100		Color tests	
Substance	BAW	2% HAc	UV	Wiesner's reagent
Unknown	82	88	dark blue	orange
Vanillin	91	67	dark blue	peach
Coniferaldehyde	89	47	light blue	cerise

It was thought that the material may have been a glucoside. Therefore, the following experiment was performed. A streak chromatogram was prepared of this substance, the band was cut-out, and the material was completely

eluted by descending chromatography with one ml. of water. To the resulting solution was added one ml. of a five per cent emulsin solution. The sample was then incubated at 33° C. for 15 hours.

The enzyme was precipitated with 8 ml. of ethanol, separated by centrifugation and washed with 3 ml. of 50 per cent ethanol. The total supernatant was evaporated to a small volume and placed on a chromatogram in two spots. After developing with BAW the strips were sprayed with p-anisidine hydrochloride and Wiesner's reagent. No spot corresponding to glucose was made visible with the p-anisidine hydrochloride spray, and the Wiesner's spray indicated that the substance was unchanged.

It was found that the unknown substance could be removed from the ib neutral solution by extraction with ethyl ether, thus freeing it from carbohydrates. Therefore, the aqueous solution was thoroughly extracted with ethyl ether, the ether solution was dried over magnesium sulfate and then evaporated under vacuum to yield a very small amount of a slightly yellow, waxy substance. Several attempts to recrystallize this substance were unsuccessful, probably because of the small amount present.

An attempt was next made to hydrolyze this compound with mineral acid. One tenth of a ml. of an approximately one per cent solution of this substance was added to an equal volume of one normal hydrochloric acid and was sealed

in a glass tube. After three hours at 105° C. the solution had become slightly yellow with a minute amount of an insoluble, gummy substance present.

When this solution was chromatographed with two per cent acetic acid and BAW, the results were inconclusive. With two per cent acetic acid there was a streak from the origin to Rf 0.58 which fluoresced in ultraviolet light, but gave no color with p-anisidine hydrochloride, diazotized p-nitroaniline or ammoniacal silver nitrate. A spot at the origin gave a brown color with both diazotized p-nitroaniline and ammoniacal silver nitrate. Another very faint spot was found at Rf 0.87. This spot was slightly blue with ultra violet light and very faintly tan with both p-anisidine hydrochloride and diazotized p-nitroaniline. This spot did not appear to be due to the original compound since it failed to give a color with either Wiesner's reagent or 2,4-dinitrophenylhydrazine.

With BAW a spot which fluoresced strongly under ultraviolet light was found at Rf 0.49. This spot gave a brown color with p-anisidine hydrochloride and diazotized p-nitroaniline. A spot at the origin gave the same color reactions with these sprays and also fluoresced with ultraviolet light. With p-anisidine hydrochloride two other very faint spots were also seen. One was a brown spot at Rf 0.18, and the other was a pink spot at Rf 0.21. These

Rf's and colors corresponded to those of glucose and arabinose, respectively.

An ultraviolet spectrum of the ib neutral, Wiesner positive substance was prepared in 95 per cent ethanol. This spectrum exhibited a maximum at 280 mμ and a minimum at 244 mμ, as shown in Figure 4. No shift occured upon addition of potassium hydroxide, thus confirming that the compound was not a phenol. The infrared spectrum of this substance is shown in Figure 5. This spectrum was determined by the potassium bromide pellet method.

It is interesting to note that re-examination of all of the other fractions from the newly formed inner bark failed to show the presence of this Wiesner positive material. However, this may have been due to the low concentration of this substance in the other fraction(s).

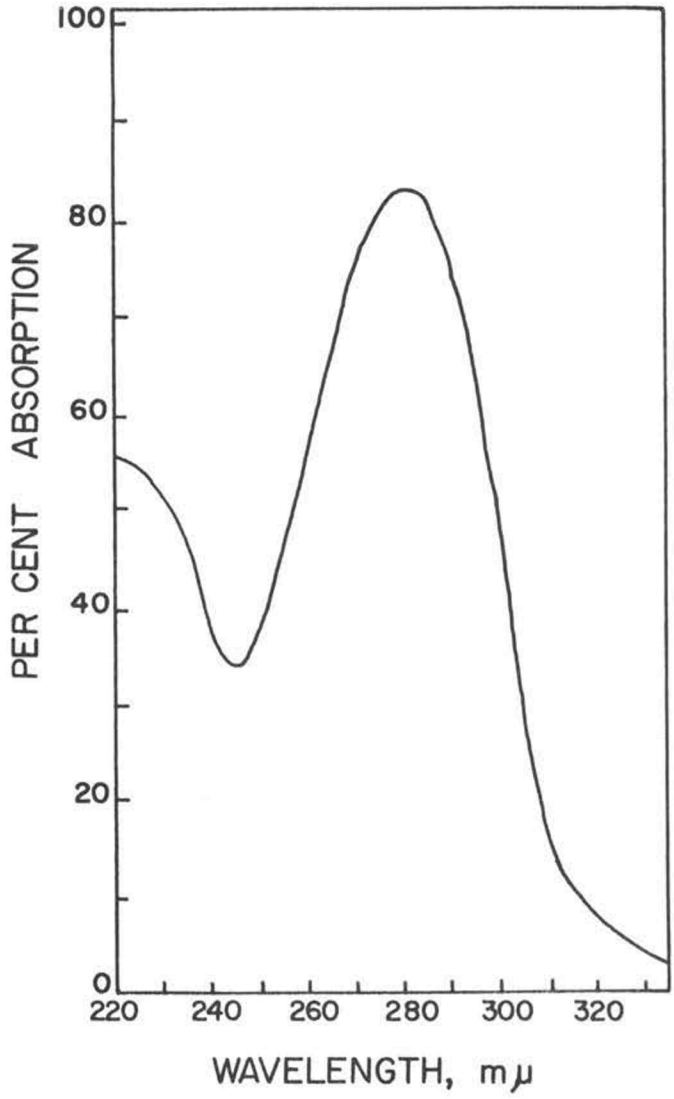


FIGURE 4. ULTRAVIOLET SPECTRUM OF WIESNER POSITIVE UNKNOWN MATERIAL

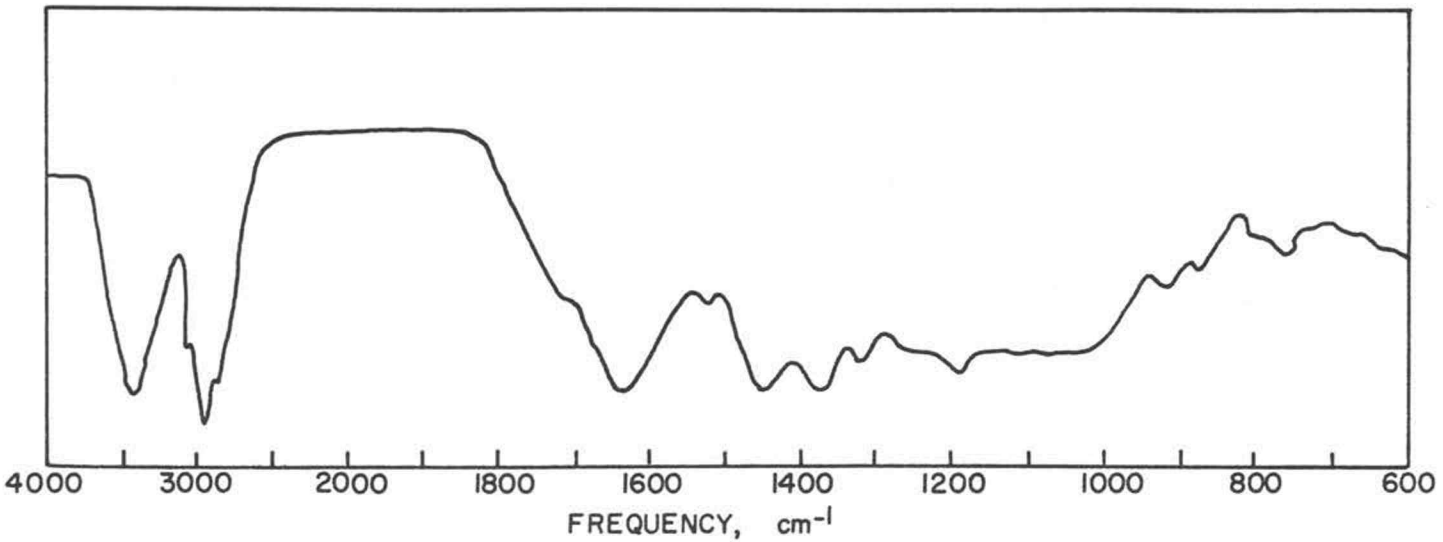


FIGURE 5. INFRARED SPECTRUM OF WIESNER POSITIVE UNKNOWN MATERIAL

RESULTS

A cursory examination of the newly formed inner bark extracts from a Douglas fir estimated to be 200 years old and several trees approximately 40 years old revealed that they were qualitatively similar. However, the older tree contained more water soluble materials, probably polyphenols, since the concentration of sugars was approximately the same for both age groups. The 40 year old trees contained much more ethyl ether soluble material than the 200 year old tree. Therefore, the younger trees were used for further work. The younger trees were also more plentiful; thus, a more representative sample could be obtained.

Analysis revealed that the hexane and methanol solubles increased from late spring to fall; while the ether solubles decreased over the same period. The amount of water solubles increased markedly between late spring and mid-summer and then decreased slightly from mid-summer to fall. These data are summarized in Table 12.

Table 12

YIELD OF EXTRACTIVES FROM NEWLY FORMED INNER BARK OF DOUGLAS FIR IN SPRING, SUMMER AND FALL

(Percentages based on moisture-free, unextracted bark)

Date sample collected	Hexane solubles	Methanol solubles	Water solubles	Ethyl ether solubles
June 6, 1959	1.21	0.75	31.2	0.92
July 21, 1960	1.57	0.93	42.0	0.74
Sept. 23, 1959	2.20	1.13	39.8	0.69

The amounts of simple sugars in mid-summer and fall were estimated paper chromatographically. While the total water solubles were found to decrease only slightly during this period, the sugars decreased greatly. Raffinose disappeared completely, while the amounts of glucose and fructose decreased from 0.7 per cent to only traces. The amount of sucrose also decreased markedly. These data are summarized in Table 13.

Table 13

ESTIMATION OF SUGARS IN NEWLY FORMED INNER BARK FROM DOUGLAS FIR IN SUMMER AND FALL

(Percentages based on moisture-free, unextracted bark)

Date sample collected	Sucrose	Glucose	Fructose	Raffinose
July 21, 1960	27.0	0.7	0.7	0.5
Sept. 23, 1959	16.0	trace	trace	-

The marcs of the newly formed inner bark, mature inner bark and outer bark were analyzed for Klason lignin and ash contents. The Klason lignin content increased from the newly formed inner bark to the outer bark. The opposite was found for the ash content of the three fractions.

Paper chromatography of the water soluble materials revealed the presence of at least ten amino acids. Aspartic acid, glutamic acid and serine were each present in

quantities exceeding 0.01 per cent of the moisture-free, unextracted bark. Seven other amino acids were present in amounts of less than 0.01 per cent each. They were proline, glycine, alanine, threonine, valine, leucine and isoleucine. The amino acids phenylalanine and tyrosine were also probably present, but in extremely small amounts.

The presence of shikimic and quinic acids was also indicated. Shikimic acid amounted to approximately 0.03 per cent of the moisture-free, unextracted bark, while the amount of quinic acid appeared to be approximately three times this value. Paper chromatography also indicated the presence of small amounts of six other unidentified hydroaromatic substances, although no phosphoric esters were indicated.

A Wiesner (phloroglucinol-hydrochloric acid) positive substance was isolated from the water soluble fraction of the newly formed inner bark. Ultraviolet and infrared spectra of this substance were prepared, and enzymatic and acid hydrolysis were attempted. However, the identity of this substance is unknown.

Examination of the methanol solubles revealed no significant differences from the water soluble, phenolic materials. This fraction was not examined further.

Analysis of the hexane soluble fraction revealed that 3 per cent of this material was in the free acid

form, 5 per cent was unsaponifiable, and 93 per cent was saponifiable. Of the latter fraction, 71 per cent was ether soluble after saponification and acidification. The remainder of the saponifiable fraction was insoluble in hexane, ether and water, but soluble in acetone.

Column chromatography of the total hexane solubles on alumina gave a crude phytosterol fraction. Crystallization from acetone of the hexane solubles gave a brown oil, whose infrared spectrum showed it to be an acid or ester. This substance did not contain free hydroxyl groups and was not aromatic.

d-Dihydroquercetin was isolated from the outer bark and appeared to be the only flavonoid compound present in this portion of the bark. Paper chromatography and color tests indicated that a small amount of d-dihydroquercetin was also present in the mature inner bark.

d-Catechin was isolated from the mature and newly formed inner bark. Another flavonoid was also present in these two fractions. Paper chromatography using several solvents indicated that this substance was l-epicatechin. However, attempts to isolate and purify this substance were unsuccessful. An infrared spectrum of this crude substance indicated that it was probably impure l-epicatechin.

DISCUSSION

The presence of sucrose, fructose and glucose in the newly formed inner bark is not unexpected as these three sugars are quite commonly found in the cambial area of trees (42). Sucrose is believed to be the principal reserve carbohydrate (32) and its predominance is quite common. Zimmerman (49) divides trees into three large arbitrary groups according to the sugar content of the sap. The groups are: (1) sap contains sucrose only, (2) sap contains raffinose, stachyose, verbascose, etc. only, and (3) sap contains a mixture of types of sugars. It appears that most trees, including Douglas fir, belong in this third group.

The function of raffinose in the cambial area is debatable; however, Zimmerman postulated that this sugar may play a role in removal of photosynthates from the sieve tubes. The absence of raffinose in the sample collected in the fall may be due to the fact that cambial activity has ceased and no further photosynthates are necessary for bark and wood synthesis. In confirmation, Grillos (17) has pointed out that Douglas fir cambial cell division ceases in August.

The low yield of glucose and fructose in the fall may also be due to the cessation of cambial activity. The conclusion could be drawn that the lignin and carbohydrate

synthesis mechanisms have ceased while the reserve carbohydrate, sucrose, remains in the sap throughout the year.

Although the yield of water soluble material from the newly formed inner bark was more than twice that from the mature inner bark, the ratio of total sugars to total water solubles was approximately the same for both fractions. This same phenomenon was also observed by Stewart (42) in Eucalyptus regnans. He suggested that the constancy of the ratio may indicate that the soluble nutrients are assimilated at the same rate during cell formation and cell growth. The ratio of total sugars to total water solubles decreased markedly in the outer bark in Douglas fir, indicating the completion of bark synthesis.

While no pentoses were found in the newly formed inner bark, a trace amount of xylose was found in the mature inner bark. Trace amounts of both xylose and arabinose were found in the outer bark. These results agree quite well with the results of Smith and Zavarin (41). They stated that xylose, arabinose and rhamnose were found in the outer bark of Douglas fir, although none of these sugars was found in the inner bark.

The total sugar content of the newly formed inner bark was found to be approximately 30 per cent, based on the moisture-free, unextracted bark. However, too much significance must not be attached to the actual figure because of the inherent error in the method of determination

(± 10 per cent) (6). It is interesting to note that the sucrose content of the cambial zone of Picea mariana was found to be 33 per cent (3).

Analysis of the marcs revealed an increase in Klason lignin content proceeding from the newly formed inner bark, through the mature inner bark and to the outer bark. Although this progressive increase was expected, the relatively high yield of Klason lignin (18.79 per cent) from the newly formed inner bark was unexpected. Stewart (42) reported a Klason lignin yield of 21 per cent for the cambial zone of Eucalyptus regnans. He showed by indirect methods (methoxyl content and yields of vanillin and syringaldehyde upon alkaline nitro benzene oxidation) that the true lignin content was less than one per cent. He attributed the high result to large quantities of waxy and protein substances.

An ash determination on the marcs revealed a marked decrease from the newly formed inner bark to the mature inner bark and a further slight decrease from the mature inner bark to the outer bark. The high yield of ash (5.19 per cent) from the newly formed inner bark is indicative of the amount of minerals needed for biochemical activity. In addition, it has been stated that approximately 50 per cent of the mineral matter is extracted with aqueous alcohol (42). Considering this fact the yield becomes very

significant. No attempt was made to identify the minerals present.

The results of the amino acid determination compare quite favorably with the results of Mugg (36) on aspen. Aspartic acid, glutamic acid and serine were present in amounts exceeding any of the other amino acids. Proline, glycine, alanine, threonine, valine, leucine and isoleucine were present in lesser amounts. Phenylalanine and tyrosine were probably present, but the very small amounts made definite conclusions by paper chromatography impossible.

The absence, or near absence, of the two aromatic amino acids is quite surprising since they are presumed to be involved in the biosynthesis of wood lignin. Mugg (36) found these two amino acids to be present in small amounts in aspen using the method of Moore and Stein (35), which is much more sensitive than the method used in this work. It is interesting to note that methionine was not present in aspen or Douglas fir, even though this amino acid is presumed to be the substance responsible for the methylation of phenols to guaiacyl and syringyl nuclei (2).

In addition to phenylalanine and tyrosine, serine and glycine have also been described as possible precursors of lignin (18). However, it must be assumed that most of the amino acid pool is used for protein synthesis rather than lignin synthesis.

Shikimic and quinic acids were found in the extract from the newly formed inner bark. These hydroaromatic acids have been shown to represent an intermediate step between carbohydrates and aromatic compounds in bacteria (31). Brown and Neish (8, 9) have shown that randomly labelled shikimic acid is transformed into labelled lignin in several hard woods. In addition, Acerbo, Nord and Schubert (1) have shown that the reaction sequence from glucose to aromatic substances in spruce is apparently identical to that of bacteria. The scheme from glucose to lignin is shown in Figure 6.

Mugg (36) and Faber (12) were unable to find any indication of either of the hydroaromatic acids in newly formed aspen wood or bark. However, no preliminary separation of the compounds was made by either of these workers. Shikimic acid has been found quite widely distributed in plants (30).

It was noted that only shikimic and quinic acid were identified in Douglas fir bark, while the biosynthetic scheme includes 5-dehydro shikimic acid and 5-dehydro quinic acid. Since six apparently hydroaromatic compounds were present but not identified, it might be assumed that the two 5-dehydro acids might be causing two of the six spots on the chromatograms. However, Hathway (22) found only one solvent which separated 5-dehydro shikimic acid from shikimic acid. When this solvent was used no evidence

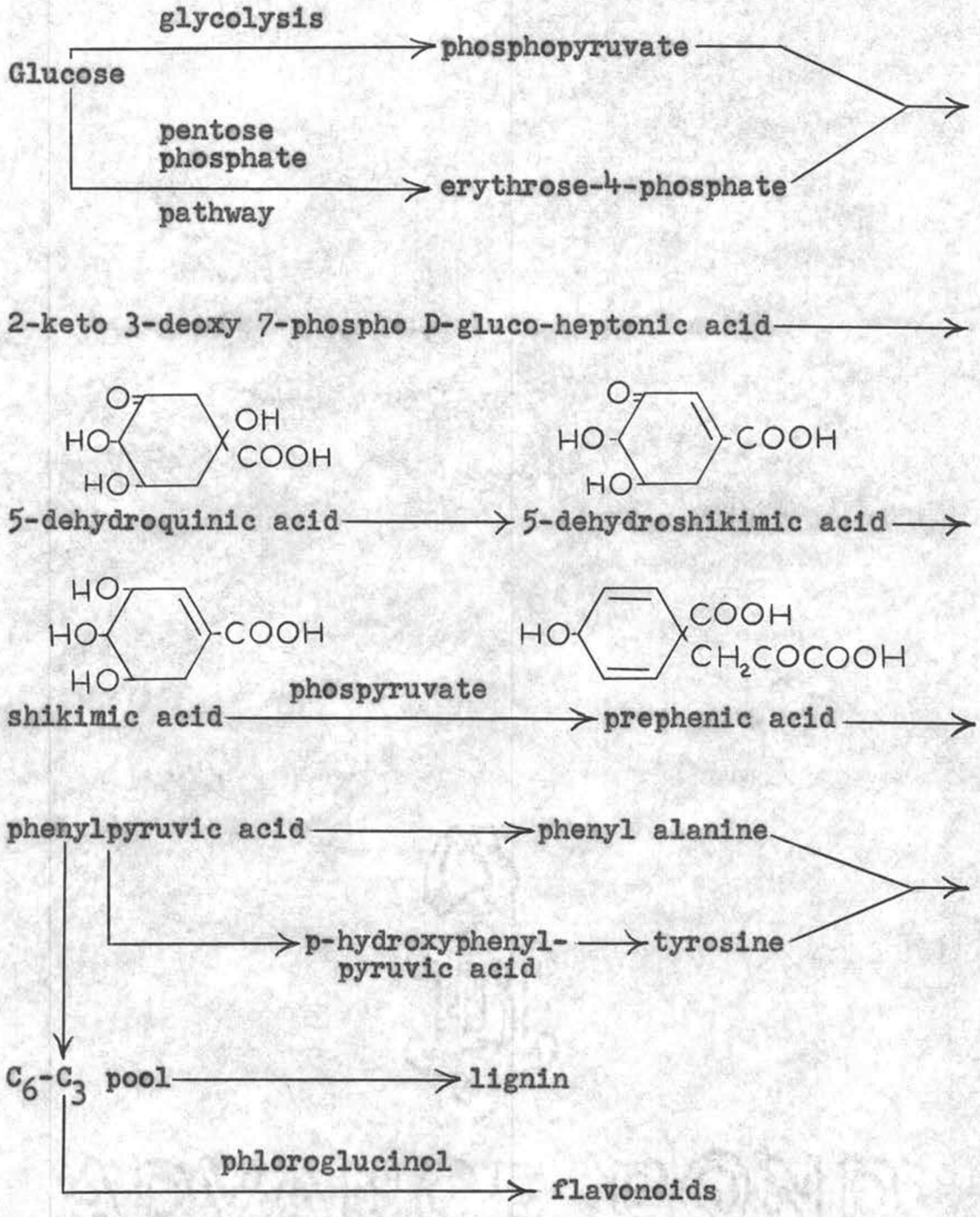


Figure 6. BIOSYNTHETIC PATHWAY FROM GLUCOSE TO LIGNIN AND FLAVONOIDS

(From Krebs and Kornberg (31) and Adler (2))

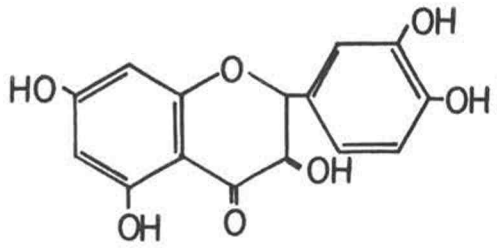
was found for the presence of 5-dehydro shikimic acid in the Douglas fir bark extract.

The indication of six other substances in this fraction was quite interesting. Due to the extremely small amounts of these substances they were not examined further. However, it is obviously possible that they may represent several as yet unknown steps in this reaction sequence.

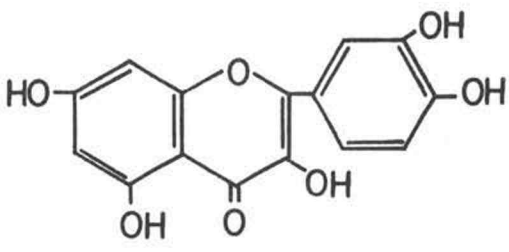
The failure to identify prephenic acid in Douglas fir is easily explained by the extreme instability of this acid. Weiss (47) has shown that prephenic acid has a half-life of only 13 hours at pH 6. This half-life decreases greatly with decreasing pH, while the pH of the water extract was less than 5.

An unsuccessful attempt was also made to indicate the presence of phenylpyruvic acid, or other aromatic acids, in the extract. It is obvious that the lack of evidence of any particular compound in a biosynthetic scheme is of little significance due to the possible highly transitory nature of that substance. However, positive evidence of any compound pre-supposed to be in a biosynthetic scheme is of much more importance.

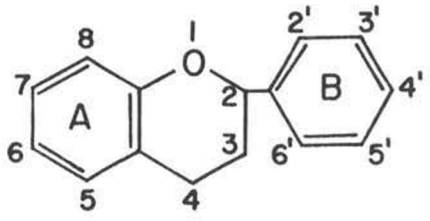
Underhill, et. al. (45) have shown that shikimic acid is also apparently a precursor of flavonoids (See Figure 7). Their work further indicated that the catechol ring and the three carbon bridge of quercetin is derived



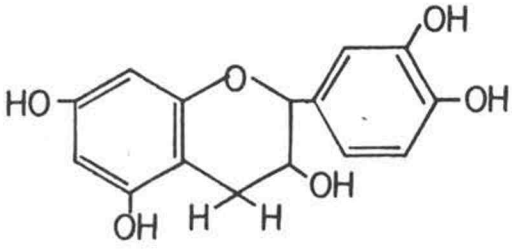
d-DIHYDROQUERCETIN



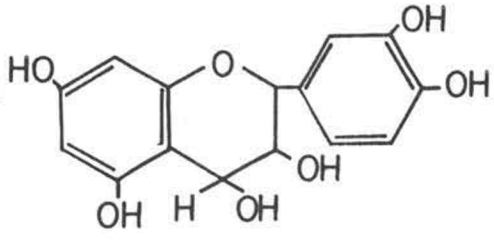
QUERCETIN



DESIGNATION OF RINGS AND NUMBERING OF FLAVONOIDS



d-CATECHIN and
l-EPICATECHIN



LEUCOCYANIDINS

FIGURE 7. STRUCTURE OF DOUGLAS FIR FLAVONOIDS

from some substance in the C_6-C_3 pool (See Figure 6). However, the phloroglucinol ring is apparently not derived from an aromatic compound formed by the shikimic acid route. Their results indicated that the phloroglucinol ring arises from acetate and not from inositol as was previously believed. They postulate that the phloroglucinol ring is formed by a cyclic trimerization of acetyl coenzyme A. This could also explain the reported presence of free phloroglucinol in Douglas fir bark (24).

Although the C_6-C_3 compound involved is unknown, Underhill (45) has shown that hydroxylation and methoxylation of the ring inhibits the incorporation of the C_6-C_3 compound. From this it may be concluded that the substitution of the "B" ring (See Figure 7) must follow dimerization. This is apparently in opposition to lignin synthesis (8, 9).

Phenylalanine was found to be an excellent precursor of quercetin, while tyrosine was much less efficient. Of several amino acids tested, glutamic acid was the most efficient after phenylalanine. It is interesting to note that glutamic acid was one of the most plentiful amino acids in the Douglas fir newly formed inner bark.

Although phosphopyruvate is generally believed to combine with shikimic acid to form C_6-C_3 compounds (31); it is interesting that serine, another amino acid found in considerable quantities in Douglas fir, could combine

with shikimic acid to form tyrosine by a more direct route. Of course, this is pure speculation with no experimental evidence.

A Wiesner positive aldehyde was found in the neutral water solubles and was extracted with ethyl ether. Paper chromatography showed that this substance was not coniferaldehyde or vanillin, both of which give positive Wiesner reactions. Color tests indicated that it did not contain free phenolic hydroxyl groups. Reference to published chromatographic data (28) revealed that this substance was not coniferin.

Its ultraviolet spectrum exhibited a maximum at 280 millimicrons and a minimum at 244 millimicrons. No shift was observed in alkaline solution. The infrared spectrum of this substance contained strong hydroxyl and apparently non-aromatic C-H stretching bands, but the carbonyl absorption band was too poorly resolved to yield much information.

The compound did not appear to be affected by β-glucosidase, but acid hydrolysis caused complete destruction into essentially unidentified products. Meager evidence was obtained indicating that glucose and arabinose may have been produced by the acid hydrolysis, but no conclusions could be drawn. The formation of resinous products by attempted acid hydrolysis is characteristic of coniferin and related substances. Due to the very small

amount of this material no further information was obtained.

At this point it might be of interest to consider the location and role of the various flavonoid compounds in Douglas fir. d-Catechin and l-epicatechin were found in the newly formed and mature inner barks, while d-dihydroquercetin was found in the outer bark. Small amounts of d-dihydroquercetin were also found in the mature inner bark. It was also noted that air drying caused an apparent increase of d-dihydroquercetin in the mature inner bark. However, this increase was estimated only in relation to the amount of catechins present.

From these results it could be concluded that d-dihydroquercetin is formed by oxidation of the catechins. However, Hergert and Goldschmid (25) have presented evidence that d-dihydroquercetin is synthesized in the form of its glucoside in the leaves (needles) of Douglas fir. They suggest that the glucoside is transported down the inner bark and to the outer bark where the aglucone is released. As they point out, the actual site of synthesis is not known, and further work must be performed before this question can be answered.

It has been generally accepted that the tannins of bark and wood are formed by the polymerization of catechins (48). Recent work has shown that tannins may be polymers of leuco-anthocyanins (38). However, the identity of the

actual monomer may not be too important for this discussion, since the catechins and leuco-anthocyanins are structurally very similar (See Figure 7). Hillis and Urbach (23) have presented evidence indicating that tannin is polymerized by bonds on the phloroglucinol ring. They also found a synthetic polymer of d-catechin to be dissimilar to tannin.

More recently Hergert (24) has described a tannin-like substance that appears to be a polymer of d-catechin and leucocyanidin. He suggested that the polymerization is accomplished by ether linkage formation. Hubbard (27) found the tannin from Douglas fir bark to contain 1.58 per cent methoxyl groups, indicating that at least a small amount of methylated nuclei were also present. Due to difficulties in purifying tannins, and the indiscriminant use of the term "tannin", it is impossible to describe the structure of tannins.

Phlobaphenes are also found in Douglas fir bark and are differentiated from tannins by their insolubility in water. They can also be formed by treating tannin with strong mineral acid and are assumed to be condensation products of the tannins.

Smith's (40) work on the phenolic acid from Douglas fir indicated a very close similarity of this substance to phlobaphenes and tannins. Among the alkaline nitrobenzene oxidation products of the phenolic acid were vanillin and protocatechualdehyde. The yield of each of these

aldehydes was less than one per cent; however, it was shown that protacatechualdehyde was very unstable under the conditions of the oxidation. Therefore, it was assumed that the yield of protocatechualdehyde represented only a small portion of the catechol nuclei present in the phenolic acid. The phenols produced by the above reaction were not identified, but it was indicated that only trihydroxy phenols were present.

Fahey(13) studied the oxidative degradation products of white fir phenolic acid. This phenolic acid was shown to be very similar to that from Douglas fir. This work indicated that the phenolic building stones consisted of phloroglucinol, catechol and guaiacyl nuclei. Two of the most significant oxidation products identified were 5-formyl vanillic acid and 5-carboxy vanillin. The presence of these two compounds indicates that a carbon-carbon bond must be present on the guaiacyl nucleus in the polymer. This bond would not be unlike that previously proposed for wood lignin (2).

It would seem likely that the phenolic acid is similar, or even identical, to tannin and phlobaphene. However, the phenolic acids are not extractable by inert solvents as are tannin and phlobaphens. This difference could be attributed to a higher molecular weight of the phenolic acid. However, molecular weight determinations are lacking.

It is also possible that the phenolic acid may be chemically combined with some cellular material. The type of bond could be an ester linkage between a carboxyl group in the cellular material (uronic acids) and a phenolic or alcoholic hydroxyl group in the phenolic acid. An ester linkage could also be formed between a hydroxyl group in the cellular material and a carboxyl group in the phenolic acid, if these groups do exist.

To the author's knowledge, no direct evidence has ever been published concerning the existence of carboxyl groups in the phenolic acids before isolation. Since the pyran ring in flavonoids is known to be very sensitive to alkaline and acid conditions (39), it would not seem unreasonable to assume that isolation cleaves the polymer at random pyran linkages, forming carboxyl groups. The decreased molecular weight would also account for the solubility of phenolic acids in organic solvents after isolation, but not before.

The presence of methoxyl groups can not be easily explained, using the catechin polymer model. However, it can be seen that the copolymerization of a small amount of coniferaldehyde, or related substances, could cause a considerable methoxyl content. Only one coniferaldehyde unit per catechin unit gives a methoxyl content of 6.6 per cent. This is well above the minimum methoxyl content of Douglas fir phenolic acid.

Another aspect that must be considered is the possible heterogeniety of the phenolic acid. Although Smith (40) concluded from paper chromatography that the phenolic acid is homogenous, it is possible that it is a mixture of closely related polymers. It has been shown that the methoxyl content of phenolic acid may vary from ca. 4 to 15 per cent. This variability was attributed to the demethylation in the outer bark, since the methoxyl content of the phenolic acid from the outer bark is much less than that from the inner bark.

In addition, one must consider the relationship between the phenolic acids isolated by caustic extraction and those isolated by dioxane-hydrochloric acid. The dioxane-hydrochloric acid phenolic acids give methoxyl yields similar to those of wood lignin; however, a Wiesner test for free coniferaldehyde groups is not given for the phenolic acid. It has been shown that part of the dioxane-hydrochloric acid bark lignin is extracted by sodium hydroxide and vice versa (40). This again points to the heterogenous nature of the phenolic acid.

In conclusion, it must be recognized that the actual structure of the bark phenolic acid is still unknown, and indeed, it is possible that each molecule may differ slightly from every other molecule. In this regard, the chemistry of wood lignin has been studied for well over a hundred years (48), but the determination of the structure

of this complex substance continues to be a major problem in the field of wood chemistry.

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