THE ISOLATION OF PIGMENT GLYCOSIDES FROM
COTTONWOOD, POPULUS MACDOUGALI, AND WESTERN
HEMLOCK, TSUGA HETEROPHYLLA

by

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INTRODUCTION

Pigment glycosides that give rise to many of the colorations found in plants occur widely distributed in nature. They are present in the leaves, flowers, fruits, and bark of many plants. Some have been isolated from woods. Link\(^1\) states that these pigment glycosides may be divided into two major classes: the plastic pigments and the pigments that exist in cell sap. The plastic pigments, according to Link, are associated with the protoplastic structure of the plant, whereas the cell sap pigments belong to the anthocyanin group of glycosides. This latter group includes the pigments that cause formation of the various shades of blue, purple, violet, mauve and magenta, and nearly all of the reds that appear in flowers, fruits, leaves, and stems of plants.

The anthocyanin group is quite comprehensive. As early as 1911 Grafe\(^2\) expressed the view that "anthocyanin must be regarded as a general term for a number of substances differing somewhat from each other in chemical constitution." Since that time the term has referred to many similar compounds that belong to the same general class.

The word anthocyan was derived from the Greek roots signifying, respectively, flower and blue. It was introduced into common usage by the botanist Marquart in 1835 to designate the blue pigment of flowers. Shortly thereafter, the belief arose
that the red and blue pigments were merely different forms of the same substance, and that the different colors were due to variations in the acidity of the cell sap. Consequently, the use of the term was extended to include all of the soluble pigments of this group. When it was found experimentally that these pigments always occur combined with sugars and thus are glycosides, the glycosidic ending "in" was added to make the present term, anthocyanin.

The non-sugar portion of the anthocyanin is called anthocyanidin. This can be separated from the sugar component by heating the glycoside in boiling dilute hydrochloric acid. Hydrolysis results, and the anthocyanidin can be separated from the solution, frequently as a chloride. In such a state it is normally insoluble in ether but generally soluble in water and alcohol. To distinguish between the anthocyanin and anthocyanidin in a mixture of the two, the following test is made: A water solution containing the substances is acidified and shaken with normal amyl alcohol. The anthocyanidin will immediately go into the amyl alcohol layer but the anthocyanin will not.

A second class of glycosides that occurs along with the anthocyanins includes the flavone and flavonol pigments. These glycosides give most of the yellow colors so often met in plant materials. They can be isolated as yellow crystalline solids. As in the case of the anthocyanins, the flavones
are generally found in combination with sugars. Hydrolysis with hydrochloric acid yields the non-sugar (or α-glucose) portion and the sugar which, in many cases, is either glucose or rhamnose.

During the past half century, considerable research has been carried out in an attempt to determine the manner in which flavones and anthocyanins are elaborated in the plant and their relation to each other. Muriel W. Onslow, one of the foremost authorities in this field, postulated that anthocyanins are formed from flavones by the action of an oxidizing enzyme. Considerable justification can be found for this view by a study of the flavones, flavonols and related anthocyanins. For example, pelargonidin differs from the flavonol kaempferol mainly in the substitution of an oxygen by hydrogen. Cyanidin and delphinidin differ respectively in the same way from the flavonols quercetin and myricetin. A brief glance at their structure shows the possibility of this relation.
Other investigators do not agree with this theory. Robinson suggests that flavones and anthocyanidins are independently synthesized from a common starting point through a transformation involving oxidation. He established the presence of colorless precursors called leuco-anthocyanidins which yield colored anthocyanidin-like pigments on treatment with hydrochloric acid in the presence of oxygen. These anthocyanidin-like pigments were sugar-free and were classified on the basis of their water solubility and their extraction from aqueous solutions with ethyl acetate. This work furnishes evidence against the theory advanced by Onslow. However, much more experimentation must be carried out before the actual elaboration of these glycosidic substances by plants can be completely understood.

Each group of pigment glycosides has a set of distinct reactions. These may be masked somewhat in impure crude extracts but when the purified pigment is obtained, the reactions are characteristic. The flavone and flavonol pigments may readily be detected in plant tissue. With bases, they give an intense yellow color which disappears on neutralization with acids. This property is almost universal in plants, thus showing how widely these groups are distributed in nature. A second test for the flavone and flavonol pigments is the formation of iron salts. These pigments give a green or brown coloration when ferric chloride is added to their
solutions. They also normally give a yellow-orange or red precipitate when lead acetate is added to their solutions.

The anthocyanins have a different series of color reactions from the flavones and flavonols. With acids they generally give a red color, while with bases they give blue or violet. However, if flavone or flavonol pigments are also present, a green color may develop with bases, due to the mixture of yellow and blue. With the lead acetate test, the anthocyanin pigments give insoluble lead salts, which have a characteristic blue color. As in the case with bases, this may be obscured by flavone and flavonol pigments and a green colored precipitate may be formed. A reaction which appears to be characteristic of the anthocyanins is the nascent hydrogen test. When these pigments are treated with nascent hydrogen, the color disappears. If the solution is then exposed to air, the color returns.

A method for differentiating between flavones and flavonols has recently been devised by Wilson. He found that flavones give a color reaction in the boric acid test, whereas flavonols do not. In this test 0.5 mg. of the purified flavone or flavonol is dissolved in 1 ml. of dry acetone. The solution is divided into two parts. To one part is added approximately 2 ml. of the boric acid-citric acid-acetone reagent and the other portion is diluted to an equal volume with a mixture of equal parts of the citric acid-acetone
solution and acetone. At the end of a few minutes the colors of the two tubes are compared. Any definitely stronger color in the tube containing the boric acid is a positive reaction, indicating the presence of a flavone. This test has been used on most of the commonly known flavones and flavonols, as well as on other similar substances which are found in plants in connection with them. In all cases only the flavones gave a positive reaction, indicating that it is a method for detecting them. The reagent must be prepared daily, since it develops a slight pink color on standing.

Anthocyanins have been divided into six main classes: pelargonidin, cyanidin, delphinidin, peonidin, malvidin, and hirsutidin. The first three are the main types, while the latter are derivatives of them. To distinguish between the groups, the Robinsons have devised the following tests:

#1. Oxidation Test. Ten per cent sodium hydroxide is added to a dilute solution of the pigment, and the solution is shaken in the presence of air. This brings about the destruction of delphinidin but leaves the other members relatively intact.

#2. Extraction with Amyl Alcohol. A layer of normal amyl alcohol is added to the solution followed by a solution of sodium acetate and ferric chloride. This gives the characteristic color reactions. However, the color is most pronounced if cyanidin is present. The violet amyl alcohol
solution changes to blue in the last stage of the reaction. This test eliminates pelargonidin, peonidin, and malvidin, as they do not give the ferric chloride test.

#3. Distribution between one per cent Hydrochloric Acid and a mixture of Anisole (5 volumes), and Ethyl Isoamyl Ether (1 volume) containing five grams of Picric Acid in 100 cc. Delphimidin is not extracted by the organic solvent layer; cyanidin is taken up to a considerable extent; whereas malvidin, peonidin, and pelargonidin are completely extracted if the solution is dilute.

#4. Distribution between one per cent Hydrochloric Acid and a mixture of Cyclohexane (1 volume) and Toluene (5 volumes). Delphimidin is not extracted; malvidin gives the organic solvent layer a blue tint; cyanidin, a pale rose tint; and peonidin and especially pelargonidin are extracted to a considerable extent.

These tests are readily applicable to crude extracts, since usually only one pigment is involved in the production of the color in the plant material extracted. Thus the classification of the anthocyanin pigment can be readily ascertained.

The extraction of the anthocyanins has been carried out in many different ways. One of the most common ways is to extract the tissue with ethyl alcohol and hydrochloric acid. Then the chloride is precipitated by addition of ether. It
is purified by dissolving in aqueous hydrochloric acid, adding alcohol, and reprecipitating the salt in ether. The final precipitation is made from alcoholic hydrochloric acid into ether. The extraction of flavones and flavonols is carried out in much the same way, though neutral alcohol is often used for the primary extract.

This brief survey of some naturally occurring pigment glycosides has furnished a basis for the experimental work described below on the pigment glycosides of the inner bark and wood of cottonwood and the wood of western hemlock.
EXPERIMENTAL PART

Pigment glycosides from three different sources were studied in this investigation. They were obtained from: (A) the inner bark of cottonwood, Populus MacDougall; (B) the wood of cottonwood; (C) the wood of western hemlock, Tsuga Heterophylla. In all cases the materials were air dried and converted to saw dust and finally to fine powders by running the saw dusts through the Wiley mill. The percentages of moisture were determined in the air dry material. Afterwards weighed amounts of these materials were extracted in large Soxhlet extractors, first with benzene and then with 95 per cent alcohol. The pigment glycosides were isolated from alcohol extracts. The exact procedures followed in each case are described below.

(A) Inner Bark of Cottonwood.

Preparation of Material

The bark used was obtained from two sources. One part was collected near Thatcher, Arizona, in July, 1946, and the other part was collected near Phoenix, Arizona, in August, 1947. In both cases, limbs of approximately six years' growth and from 5 to 7 inches in diameter were used. The bark was peeled from the limbs and the outer bark was stripped away with a sharp knife. After drying in the air, the inner bark was converted to a powder.
Benzene Extract

Samples varying in size from 350 to 650 g. were placed in cotton bags in a large Soxhlet apparatus and extracted with benzene for approximately 70 hours. This gave a yellowish-brown solution. When the benzene was distilled off, there remained a brownish oil that amounted to approximately 1.60 per cent of the bark used. The examination of this oil is the subject of another thesis. 10

Alcohol Extract

After extraction with benzene, the finely powdered material was returned to the bag and extracted with 95 per cent ethanol in the large Soxhlet for approximately 70 hours, or until the alcohol removed no further color. The reddish-brown alcohol solution was filtered to remove any powder that might have been drawn over. It was then concentrated in vacuo to a reddish-brown gum. The yield of this was 11.8 per cent of the moisture-free sample.

Isolation of the Glycoside

The gum was dissolved in water and saturated with sodium chloride to remove any tannins. This precipitated a reddish-brown gum. After standing for two days, the gum was filtered from the light yellow solution. When a little of the brown gum was treated with a solution of sodium hydroxide, a deep yellow color developed. As more of the gum was added, the solution became darker, and finally changed to reddish-brown. It evidently had acidic properties since it was readily solu-
ble in dilute alkali but practically insoluble in water.

The precipitate was washed with a saturated solution of sodium chloride and this was added to the original filtrate. The filtrate was concentrated to 200 cc. and extracted three times with 250 cc. portions of ethyl acetate. This gave a deep yellow color to the ethyl acetate solution and left the water solution with a brownish tinge. The ethyl acetate solution was dried over anhydrous sodium sulfate to remove any traces of water, since water interferes with the precipitation of the glycoside. The acetate solution was filtered and concentrated in vacuo to approximately 150 cc. This gave a deep yellow solution which was dried further over sodium sulfate for several hours. This solution was filtered and run in a very fine stream into seven volumes of low boiling petroleum ether which was constantly stirred automatically. This gave a flocculent yellowish-brown precipitate which was allowed to stand overnight for coagulation. The petroleum ether was siphoned off and replaced by 150 cc. of ligroin. The ligroin was used since it has a higher boiling point than the petroleum ether and does not cause condensation of moisture during isolation of the solid. The precipitate was transferred to a centrifuge bottle. After centrifuging, the material was a light yellow brown solid that seemed to be almost crystalline. The ligroin was poured off and a little petroleum ether added to wash the precipitate. This was centrifuged and the petroleum ether
poured off, the centrifuge bottle and contents were immediately placed in a vacuum desiccator to prevent moisture condensation. It was left in the desiccator for several days with frequent air changes to complete the removal of the petroleum ether. The yield of semi-crystalline solid was approximately 0.5 per cent of the original bark used.

**Purification of the Glycoside**

Approximately two grams of the glycoside were dissolved in 125 cc. of water. This gave a yellowish-brown solution which was extracted three times with 250 cc. portions of ethyl acetate. The resulting yellow ethyl acetate solution was dried over sodium sulfate for two days and concentrated in vacuo to 150 cc. This was again dried over sodium sulfate for three hours and then run with constant stirring into seven volumes of petroleum ether. After standing overnight, the flocculent yellow-white precipitate was washed with ligroin and petroleum ether as before and centrifuged. The petroleum ether was poured off and the precipitate placed in a vacuum desiccator for drying. The yield was approximately one gram of an almost chalk white semi-crystalline material or approximately 50 per cent of the original glycoside.

**Hydrolysis of the purified glycoside.**

In order to establish the presence of a glycoside, four tenths of a gram of the purified precipitate was dissolved in 20 cc. of water and 3 cc. of hydrochloric acid were added.
The mixture was heated on a water bath for five hours, yielding a brownish black precipitate and a brown colored solution. The precipitate was filtered off. The solution was neutralized carefully with sodium hydroxide and two drops excess hydrochloric acid were added. When the solution was slightly basic, it changed to deep red but returned to light brown when the excess hydrochloric acid was added. The solution was concentrated in vacuo to approximately 5 cc. Then 10 cc. of normal amyl alcohol were added to extract the remaining a-glucose. This left a colorless water solution of the sugar and a reddish-brown amyl alcohol solution of the a-glucose.

**Preparation of Glycoside for Osazone Test**

Two grams of the original glycoside from the first four samples were dissolved in 40 cc. of two per cent hydrochloric acid, yielding a yellow solution. This was placed in a flask under a reflux and heated to boiling for fifty minutes over the free flame. At the end of this time, a black semi-solid mass, the a-glucose portion, was floating in the solution. The solution was allowed to cool and the black material, which had solidified, was filtered off. The solution was extracted with 25 cc. of normal amyl alcohol to remove any a-glucose which might remain in the solution. During this treatment the amyl alcohol layer changed to a yellow-brown, thus showing that some of the a-glucose was soluble in the acidic solution.
The amyl alcohol was decanted and the water solution was just neutralized with sodium hydroxide and then made acidic with acetic acid.

**The Osazone Test for the Sugar**

The latter water solution, slightly acidified with acetic acid, was concentrated to approximately 20 cc. to remove the last traces of amyl alcohol. It was mixed with three grams of phenyl hydrazine hydrochloride and four and one-half grams of sodium acetate and placed in a boiling water bath. After ten minutes, a yellow precipitate began to settle out from the hot solution. Heating was continued for thirty minutes and the solution was allowed to remain in the water bath and cool slowly overnight. The precipitate consisted of long needle-like crystals. When examined under the microscope, these crystals were very similar to glucosazone. This indicates that the sugar portion of the glycoside is d-glucose, d-fructose, or d-mannose. However, a mixed melting point was made as follows: The osazone from the glycoside was filtered off and recrystallized from 50 per cent alcohol. Glucosazone was prepared from pure glucose. Its crystal structure was exactly like that obtained from the glycoside. It was also recrystallized from 50 per cent alcohol. Both the pure glucosazone and that prepared from the sugar of the glycoside were placed in a vacuum desiccator and dried for two weeks. The melting points of the osazones were then determined to be as follows:
Melting point of pure glucosazone........205°
Melting point of osazone from glycoside...204°
Melting point of mixed compounds..........204-205°

Since the melting point was not changed by mixing the pure glucosazone with the osazone from the glycoside, the sugar present must be d-glucose, d-fructose, or d-mannose.
To distinguish between these sugars, the Seliwanoff test\(^\text{14}\) for keto hexoses was made. This gave a negative result, thus eliminating fructose. The phenyl hydrazone test\(^\text{14}\) for d-mannose was made with negative results, thus eliminating that sugar. Therefore the sugar present was proved to be d-glucose.

**The Specific Rotation**

The specific rotation of the original glycoside was determined in a two decimeter tube, using 50 per cent alcohol as the solvent. The glycoside prepared from the bark from Thatcher shows \([\alpha]_b^{\text{ir}}=-59\). After purification of this glycoside, it showed \([\alpha]_b^{\text{ir}}=-56.6\). However, the glycoside prepared from the bark from Phoenix showed a somewhat lower rotation. This latter unpurified glycoside showed \([\alpha]_b^{\text{ir}}=-35\). After purification, it showed \([\alpha]_b^{\text{ir}}=-39.5\). This difference in the specific rotation of the glycoside from the two locations may be due to differences in the time of collection of the bark or to the effect of aging in the bark collected in 1946.
Determination of the Per Cent Sugar in the Glycoside

The quantitative sugar determination was carried out as follows: 0.2677 grams of purified glycoside was hydrolyzed as described above. However, the hydrolysis was carried out for one hour to insure completeness of the reaction. The a-glucosone portion was filtered off and the solution extracted with amyl alcohol. The alcohol layer was poured off and the water solution was diluted to 100 cc. in a volumetric flask. Then 25 cc. portions were analysed for per cent of sugar by the Schaalfer Hartman method. This 25 cc. of sugar solution was found to be equivalent to 3.30 cc. of 0.046 Normal sodium thiosulfate. This was equivalent to 44.024 mg. of copper or 20.6 mg. of glucose. Thus the total 100 cc. would contain 83.4 mg. of glucose or 30.9 per cent of d-glucose in the purified glycoside. From this the approximate molecular weight of the a-glucosone portion can be calculated if we assume that there is one molecule of glucose to one molecule of a-glucosone. This would give the molecular weight of the a-glucosone as approximately 362 and of the glycoside as 524.

Methoxyl Determination

The per cent methoxyl in the glycoside was determined by the Zeisel method. A complete description of the apparatus and procedure was given by Schmich. Calculations were made by the following formula:

\[
\% \text{Methoxyl} = \frac{\text{cc. of thiosulfate} \times \text{normality} \times 31.03 \times 100}{\text{weight of sample} \times 6 \times 1000}
\]
The glycoside from cottonwood bark contained 5.87 per cent methoxyl. This value was used in calculating its molecular weight by assuming one methoxyl group to each molecule of glycoside. Calculations on this basis give a molecular weight of 528. This compares favorably with the molecular weight of 524 obtained by sugar titration.

Comparison of Extracts of Inner Bark from Phoenix and Thatcher

The results obtained on examination of the inner bark of cottonwood are listed in Table I below.

**TABLE I**

Comparison of the Bark from Phoenix and Thatcher

<table>
<thead>
<tr>
<th></th>
<th>Yield of Benzene Alcohol Extract</th>
<th>Yield of Alcohol Extract</th>
<th>Per Cent Moisture</th>
<th>[α]₀^25 of the Glycoside</th>
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<tbody>
<tr>
<td>Phoenix</td>
<td>1.54%</td>
<td>9.34%</td>
<td>6.0</td>
<td>-39.3</td>
</tr>
<tr>
<td>Thatcher</td>
<td>1.70%</td>
<td>11.80%</td>
<td>5.9</td>
<td>-56.5</td>
</tr>
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</table>

Discussion of Table I

The results in Table I show very little difference between the bark from Phoenix and Thatcher. The percentage of alcohol extract and the specific rotation of the glycoside are somewhat higher for the bark collected near Thatcher. No explanation can be given for these differences. However, the bark from Thatcher was collected in July, 1946, and that from Phoenix in August, 1947. Thus the effect of aging as well as the dif-
ferent time of collection might be contributing factors. 

**Qualitative Tests for the Class to which the Glycoside Belongs**

A little of the purified material was dissolved in 25 cc. of water. This solution was divided into four equal parts. To the first part were added four drops of 25 per cent sodium hydroxide. This gave a deep yellow colored solution. To the second part were added four drops of concentrated hydrochloric acid. This test gave no reaction. To the third part were added four drops of ferric chloride. A deep green color was developed. To the fourth part were added four drops of a basic lead acetate solution. A light yellow precipitate was formed. These tests all indicate the presence of a flavone-flavonol pigment. The tests were characteristic and not appreciably marked by impurities, thus indicating that the substance is almost a pure flavone-flavonol glycoside.

**Summary of the results on the Alcohol Extract from Cottonwood Bark**

The color tests on this extract shows the definite presence of a flavone-flavonol pigment. Calculations based on the per cent methoxyl and per cent sugar indicate that there is one methoxyl group for every sugar group present in the glycoside. This one methoxyl group must be on the a-glucose portion since hydrolysis results in the formation of methoxyl-free glucose and the a-glucose.

A brief glance at the empirical formulae of the various flavone-flavonols proves interesting. Kaempferol (C_{15}H_{10}O_{6})
and Myricetin (C15H10O8) are representative of this group and their structures are shown in the introduction. Their molecular weights are respectively 286 and 318. If one of their hydroxyls were replaced by a methoxyl and another substituted by a glucose molecule to give the glycoside, the resulting molecular weights would be 462 and 494. This approximates the two values of 524 and 528 found for the glycoside from the inner bark by methoxyl and sugar determination. It thereby justifies the conclusion that the glycoside present is a mono-methoxyl, mono-glucosido flavone-flavonol pigment of high molecular weight. Other flavone-flavonol derivatives are known with higher molecular weights than that of myricetin. Thus the α-glucone portion found could readily be one of these. However, the exact nature of the α-glucone portion is not known, since further experimentation was not carried out.

(B) Cottonwood Sapwood

Preparation of the Wood for Extraction

The wood used in this investigation was from the ordinary variety of cottonwood which is found in Arizona, Populus MacDougalii. The limbs used were five or six years old and three to five inches in diameter. The bark was peeled off and the wood was dried in air. When completely dry, the limbs were shredded with a planer down to the heart-wood. These shavings were ground to a fine powder by running through a Wiley mill.
Benzene Extract

The benzene extract was prepared as previously described. It was pale yellow and the yield was .64 per cent of the wood used.

Alcohol Extract

The alcohol extract was prepared as previously described. The yield was 2.74 per cent of the dry wood used.

Separation of the Alcohol Extract into Fractions

The crude solid alcohol extract was washed with 200 cc. of water. Very little of the material dissolved. When common salt was added to the water extract to precipitate possible tannins, a very small amount of light brown precipitate formed. This was filtered off and the water solution carried through the same procedure that was used on the inner bark. A very small amount of white powdery material was obtained. As a result, the solid alcohol extract was separated into fractions as described below.

The alcohol extract of cottonwood was washed with 250 cc. of water and allowed to stand overnight in the water. The solution was filtered at the pump from the reddish-brown solid glycosides. After drying, the material was dissolved in 200 cc. of 95 per cent alcohol. This gave a brownish-red solution. To the solution were added 400 cc. of water. This caused a part of the material to precipitate out. Three cc. of hydrochloric acid were added to coagulate the precipitate and the solution was allowed to stand for several hours. It was then separated by centrifuging into a deep red solid, Fraction A, and a brownish-red solution.
After drying, Fraction A weighed 3.7 g. and was a deep reddish-brown solid, which amounted to 45.1 per cent of the original alcohol extract. Fraction A was powdered and shaken with 120 cc. of ethyl acetate. The part that remained insoluble was called Fraction A₁. This was centrifuged out, dried, and found to weigh 2.4 g., or 29.3 per cent of the original alcohol extract. The portion of Fraction A which was soluble in ethyl acetate was dried over sodium sulfate for several hours. After filtering, it was run with constant stirring into six volumes of petroleum ether. The precipitate which formed was allowed to stand overnight. It was then centrifuged out and isolated in the same way as the glycoside from the cottonwood bark. The material was a light reddish-brown solid which is called Fraction A₂. It amounted to 12.4 per cent of the original alcohol extract.

The filtrate from Fraction A was placed in a two-liter beaker and 800 cc. of water were added. This was six volumes of water to one volume of alcohol. The addition of water caused the formation of a light-brown precipitate, which, after coagulation, was centrifuged out. This part of the material, which consisted of 28.9 per cent of the alcohol extract, was called Fraction B. It, like Fraction A, was divided into two parts: Fraction B₁, the part of B that was insoluble in ethyl acetate; and Fraction B₂, the part of B that was soluble in ethyl acetate but precipitated by addition to six volumes of petroleum ether. Fraction B₁ was a brownish-
red solid, and consisted of 17.1 per cent of the alcohol extract. Fraction B2 was almost chalk-white and contained 8.5 per cent of the original alcohol extract.

The filtrate from Fraction B was light brown. It was neutralized with sodium hydroxide before concentrating in order to prevent hydrolysis. When the solution was on the basic side, it was light red. It was then made slightly acidic with acetic acid, regaining its original brown color. To this solution were added the 250 cc. of water solution obtained in the original treatment of the gum. This gave a total volume of 1600 cc. It was concentrated in vacuo. As the solution became more concentrated, the color deepened to yellow-orange and a colloidal suspension was formed. When the volume was approximately 350 cc., the solution was centrifuged to remove the material that had precipitated. This material was called Fraction C. It was soluble in a solution containing 1 volume of alcohol to 6 volumes of water. It was a brownish-red powder consisting of 18.3 per cent of the alcohol extract. Fraction C was divided into two parts: Fraction C1 and Fraction C2. The former was insoluble in ethyl acetate. It was a fluffy light red powder amounting to 7.4 per cent of the alcohol extract. Fraction C2 was soluble in ethyl acetate but precipitated from six volumes of petroleum ether. It was a white powder that made up 7.3 per cent of the alcohol extract.
The 350 cc. of solution from which Fraction C had been precipitated was extracted three times with 250 cc. portions of ethyl acetate. This gave a yellow color to the acetate and left the water solution light brown. The ethyl acetate solution was dried over sodium sulfate for several hours and concentrated to 150 cc. Again it was dried and then run with constant stirring into five volumes of petroleum ether. The flocculent precipitate that formed was allowed to stand overnight, centrifuged, washed, and placed in a vacuum desiccator to dry. It was a white powder called Fraction D. It amounted to 9.7 per cent of the alcohol extract.

Methods of Analysis of the Fractions

Small amounts of the seven fractions into which the alcohol extract had been separated were used in the following series of tests and analyses.

Melting Point

The melting point was determined by placing a little of the material in a melting point tube and attaching this tube to a calibrated thermometer. The tube and thermometer were immersed in an oil bath and the temperature gradually raised until the sample melted.

Specific Rotation

The specific rotations of the different fractions were determined in either one or two decimeter tubes, depending upon the color of the solution. The solvent used was 95 per cent alcohol, since some of the fractions were insoluble in water.
Hydrolysis

The material was dissolved in alcohol and a little water added. Then sufficient hydrochloric acid was added to make the solution approximately 2 per cent acid and the solution was heated under a reflux. As the alcohol boiled away, water was added to replace it. This process was continued for one hour over the free flame, giving in all cases an insoluble black a-glucose portion and glucose.

Quantitative Sugar Determination

A carefully weighed sample was hydrolyzed as above. The excess alcohol was boiled away and the a-glucose portion was filtered off. The water solution was extracted with normal amyl alcohol to remove any soluble a-glucose. This left the water solution almost colorless. It was diluted to 100 cc. in a volumetric flask, and 25 cc. portions were used for sugar determinations by the Schaffer-Hartman method. From the results, the per cent sugar was calculated.

Methoxyl Determination

The per cent methoxyl was determined by the Zeisel Method previously described.

Results of the Analyses

The results of the analyses obtained on the fractions of alcohol extract from cottonwood sapwood are given in Table II.
### TABLE II

Analyses of Fractions
Of the Alcohol Extract

<table>
<thead>
<tr>
<th>Melting Point</th>
<th>Specific Rotation</th>
<th>Per Cent Sugar</th>
<th>Per Cent Methoxyl</th>
<th>Molecular Weight from Methoxyl</th>
<th>Approx. No. Methoxyls Per Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction A1</td>
<td>Above 260°</td>
<td>-126</td>
<td>1.75</td>
<td>13.4</td>
<td>226</td>
</tr>
<tr>
<td>Fraction A2</td>
<td>107 - 112°</td>
<td>-44.8</td>
<td>3.35</td>
<td>10.8</td>
<td>279</td>
</tr>
<tr>
<td>Fraction B1</td>
<td>Above 260°</td>
<td>-34.5</td>
<td>3.98</td>
<td>12.9</td>
<td>230</td>
</tr>
<tr>
<td>Fraction B2</td>
<td>89 - 92°</td>
<td>-21.8</td>
<td>5.12</td>
<td>12.1</td>
<td>244</td>
</tr>
<tr>
<td>Fraction C1</td>
<td>149 - 153°</td>
<td>-42.4</td>
<td>7.66</td>
<td>12.4</td>
<td>238</td>
</tr>
<tr>
<td>Fraction C2</td>
<td>92 - 95°</td>
<td>-24.3</td>
<td>6.74</td>
<td>12.3</td>
<td>236</td>
</tr>
<tr>
<td>Fraction D</td>
<td>71 - 74°</td>
<td>-15.4</td>
<td>5.04</td>
<td>13.8</td>
<td>218</td>
</tr>
</tbody>
</table>
Classification Tests

In order to classify the fractions, another series of tests were run as follows: A little of the material was dissolved in 25 cc. of 95 per cent alcohol. This solution was divided into four equal parts. To Part 1 were added four drops of 25 per cent sodium hydroxide. To Part 2 were added four drops of concentrated hydrochloric acid. To Part 3 were added four drops of ferric chloride solution. To Part 4 were added four drops of a basic lead acetate solution. The results of these tests are summarized in Table III.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sodium Hydroxide</th>
<th>Hydrochloric Acid Test</th>
<th>Ferric Chloride Test</th>
<th>Lead Acetate Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1$</td>
<td>green</td>
<td>light red*</td>
<td>dark green-brown</td>
<td>brown ppt.</td>
</tr>
<tr>
<td>$A_2$</td>
<td>lt. yellow</td>
<td>colorless</td>
<td>yellow-brown</td>
<td>yellow ppt.</td>
</tr>
<tr>
<td>$B_1$</td>
<td>green-brown</td>
<td>light red*</td>
<td>deep green ppt.</td>
<td>brown-green ppt.</td>
</tr>
<tr>
<td>$B_2$</td>
<td>yellow</td>
<td>colorless</td>
<td>green-brown</td>
<td>yellow ppt.</td>
</tr>
<tr>
<td>$C_1$</td>
<td>yellow</td>
<td>trace of red</td>
<td>green-brown</td>
<td>brown ppt.</td>
</tr>
<tr>
<td>$C_2$</td>
<td>bright yellow</td>
<td>colorless</td>
<td>light green</td>
<td>yellow ppt.</td>
</tr>
<tr>
<td>$D$</td>
<td>bright yellow</td>
<td>colorless</td>
<td>light green</td>
<td>yellow ppt.</td>
</tr>
</tbody>
</table>

*Red color is removed when treated with zinc and hydrochloric acid. Upon standing in air, red color returns.
Summary of the Analyses of the Fractions of the Alcohol Extract

The color tests, Table III, indicate that the alcohol extract is a mixture of two classes of pigments, the anthocyanins and the flavone-flavonols. The tests given by Fractions A₁ and B₁ prove that these fractions contain an anthocyanin. They are the least water-soluble parts of the extract. Tests given by Fractions A₂, B₂, C₁, C₂, and D show the presence of flavone-flavonol pigments.

The methoxyl groups must all be located on the a-glucone portion of the glycoside. This was shown by the hydrolysis of the material which yielded methoxyl-free glucose and the a-glucone portion. The ratio between the methoxyl and sugar groups present in the fractions of the alcohol extract are quite different from the 1 to 1 ratio found in the glycoside from the inner bark. In the various fractions, the ratio varies from 32 methoxyls to 1 glucose in the less soluble fraction to a 9 to 1 ratio in the more soluble parts.

The molecular weights of the sugar free a-glucone portion figured on a basis of one methoxyl to one a-glucone unit varies from 218 to 279. This compares favorably with the molecular weights of the well-known flavone-flavonol and anthocyanin groups, which commonly range from 212 to 318. The rather striking agreement between these calculated and theoretical molecular weights leads to the conclusion that in the wood, as in the bark, there is one methoxyl
group to each a-glucone portion. To make possible agreement with the sugar determinations, we must assume that much of the material is in the sugar free state. Thus, since there is one glucose to every 32 methoxyls in Fraction A and only one methoxyl to each a-glucone unit, it implies that there are approximately 31 a-glucone units to each complete glycoside. Likewise, in Fraction A, there would be 16 a-glucone units per glycoside, etc. Thus we must conclude that the alcohol extract contains a mixture of various flavone-flavonol and anthocyanidin pigments together with a small fraction of flavone-flavonol and anthocyanin glycosides.

(C) Wood of Western Hemlock

Preliminary Treatment

The wood used was obtained from The Institute of Paper Chemistry, Appleton, Wisconsin. It was air dried, after removing the bark, and converted to a fine powder.

Benzene Extract

The finely powdered wood was extracted with benzene as previously described. This gave a light yellow solution which on concentration amounted to 0.44 per cent of the original air dried wood.

Alcohol Extract

After extraction with benzene, the finely powdered material was extracted with 95 per cent alcohol for 18 to 20 hours or until the alcohol came over colorless from the
extractor. The deep red solution was filtered and concentrated in vacuo. This gave a reddish-brown gummy material which made up 4.02 per cent of the air dried wood.

**Division of Alcohol Extract into Fractions**

The dry alcohol extract was mixed with water and allowed to stand several hours. The solution was filtered from a yellow-brown solid and reserved for later treatment. The solid material was dissolved in 150 cc. of cold alcohol. After standing, most of the solid dissolved, giving a deep red-brown solution. This solution was filtered to remove any insoluble part and two volumes of water were added. This caused a dark colored precipitate to form, which was coagulated by addition of a trace of hydrochloric acid. After standing for one hour, the solution was centrifuged to remove the precipitate. This insoluble material was a fine red powder and amounted to 51.9 per cent of the alcohol extract. It was called Fraction A.

The solution from Fraction A had a slightly pink-yellow color. To it was added enough water to make the water to alcohol ratio 8 to 1. This precipitated Fraction B which was centrifuged from the solution as a light red material. Fraction B was that part of the alcohol extract which was soluble in a ratio of 2 volumes of water to 1 volume of alcohol, but insoluble in a ratio of 8 to 1. After drying, the powder had a light color and amounted to 22.2 per cent of the alcohol extract.
The solution from Fraction B was neutralized with sodium hydroxide and then faintly acidified with acetic acid. To it was added the water solution from the original washing of the extract. This total solution was concentrated in vacuo, the color becoming deeper with concentration. When the alcohol had distilled off, a light precipitate formed in the remaining water solution. This was centrifuged out, dried, and called Fraction C. It amounted to 11.1 per cent of the alcohol extract.

The solution was concentrated to approximately 250 cc. and extracted with three 250 cc. portions of ethyl acetate. This gave a slight yellow color to the acetate solution and left the water solution a light brown. The ethyl acetate solution was dried several hours over sodium sulfate and concentrated in vacuo to 150 cc. This solution was again dried and run, with constant stirring, into 5 volumes of petroleum ether. A flocculent precipitate formed which was allowed to stand overnight. This precipitate was centrifuged, washed with ligroin and petroleum ether, and placed immediately in a vacuum desiccator to dry. This light yellow material was called Fraction D and amounted to 14.8 per cent of the alcohol extract.

Analysis of Material Extracted with Alcohol

A series of determinations were run on the four fractions obtained from the western hemlock. These were melting
point, specific rotation, hydrolysis, quantitative sugar and methoxyl determinations, all of which were described above. The results of these tests are listed below in Table IV, on page 34.

Classification Tests.

The series of color tests previously described was made on these fractions in order to classify them. The reactions and results of these tests are given in Table V, on page 35.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Melting Point</th>
<th>Specific Rotation</th>
<th>Per Cent Sugar</th>
<th>Per Cent Methoxyl</th>
<th>Molecular Weight From Methoxyl</th>
<th>Approx. No. Methoxyls Per Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>138 - 142°</td>
<td>-105</td>
<td>1.19</td>
<td>17.42</td>
<td>176</td>
<td>76</td>
</tr>
<tr>
<td>B</td>
<td>103 - 107°</td>
<td>-41.2</td>
<td>2.57</td>
<td>10.78</td>
<td>130</td>
<td>34</td>
</tr>
<tr>
<td>C</td>
<td>115 - 118°</td>
<td>-46.3</td>
<td>2.24</td>
<td>15.28</td>
<td>138</td>
<td>30</td>
</tr>
<tr>
<td>D</td>
<td>82 - 85°</td>
<td>-19.6</td>
<td>3.89</td>
<td>16.11</td>
<td>13.5</td>
<td>22</td>
</tr>
<tr>
<td>Fraction</td>
<td>Sodium Hydroxide</td>
<td>Hydrochloric Acid Test</td>
<td>Ferric Chloride Test</td>
<td>Lead Acetate Test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>deep green with ppt.</td>
<td>deep red *</td>
<td>green-blue</td>
<td>green-brown ppt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>brown-green with ppt.</td>
<td>light red *</td>
<td>green-blue</td>
<td>brown ppt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>yellow</td>
<td>colorless</td>
<td>green-brown</td>
<td>yellow ppt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>yellow</td>
<td>colorless</td>
<td>green</td>
<td>yellow ppt.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Red color is removed when treated with zinc and Hydrochloric acid. Upon standing in air, red color returns.
Summary of the Results on the Fractions of Alcohol Extract

The color test, Table V, indicate that the alcohol extract of western hemlock contains both anthocyanin and flavone-flavonol pigments. Fractions A and B, the less water soluble parts of the extract, give characteristic anthocyanin reactions. Fractions C and D show pronounced flavone-flavonol properties. Flavone-flavonol pigments may also be present to some extent in Fractions A and B since their reactions would be masked by the color reactions of the anthocyanin.

The methoxyl groups in the extract from this wood as in those from the cottonwood must be located on the a-glucose unit, since methoxyl-free glucose was obtained upon hydrolysis. The extremely high methoxyl to glucose ratio is somewhat higher than that found in cottonwood. Calculations based on one methoxyl to each molecule of pigment give molecular weights ranging from 176 to 185. Since these values are lower than the average molecular weights of the known flavones and anthocyanins, we can conclude that a mixture of mono- and di-methylated units are present. If this is correct, the fractions must be made up of a mixture of sugar-free units and a small amount of the glycoside.
The results of this investigation indicate that the simpler pigment glycosides are found in the inner bark of the tree. In the inner bark, only one class of glycoside, the flavone-flavonols, was found. This could be isolated in almost pure form as shown by the close agreement between the molecular weights found by sugar titration and methoxyl determination. Analysis of this pigment glycoside gave evidence indicating it was a mono-methoxyl, mono-glucosido flavone-flavonol.

The pigment glycosides found in the two woods were complex mixtures. This is shown by the presence of both flavone-flavonol and anthocyanin pigments in cottonwood and western hemlock. Another indication is the difficulty encountered in separating the pigment glycosides. This was comparatively easy in the bark, but in the woods a mixture was obtained in every fraction.

The study would not prove or disprove either Onslow's or Robinson's theory on the elaboration of these glycosides in plants. However, it does show that in cottonwood, the living part of the plant contains only the flavone-flavonol groups whereas the wood contains, in addition, anthocyanins. This would leave open the possibility that a part of the simpler flavone-flavonols might be transformed into the anthocyanins during the aging of the wood.
cell. The low sugar content of the wood pigment suggests that this aging process removes by far the greater portion of the glycosidic glucose, leaving a mixture high in a-glucose pigment and low in glycoside. The nature of products from western hemlock corresponded so closely with that of cottonwood as to substantiate the above conclusions.
BIBLIOGRAPHY


3. Ibid., p. 93.


15. Ibid., p. 204.