

#1932 THE INSTITUTE OF PAPER CHEMISTRY
(Lignin & Wood Study)
Project Reports (1)

PROJECT REPORT FORM

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Dr. Pearl
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PROJECT NO. 1932
COOPERATOR Institute
REPORT NO. 1
DATE May 18, 1956
NOTE BOOK 1359
PAGE 94 - 113 and 118 - 122
SIGNED *Donald L. Beyer*
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CAUSTIC HYDROLYSIS OF WHOLE AND EXTRACTED ASPEN SAWDUST

ACID HYDROLYSIS OF NATIVE ASPEN LIGNIN

The two caustic hydrolyses were run using identical procedures. A 100 gram O.D. sample was boiled under reflux in 3000cc. of 4% sodium hydroxide solution.

When cool, the mixture was filtered and washed with water. The sawdust was washed with acetic acid, followed with an ethanol wash and finally an ether wash. The sawdust was then left to air dry. When dry, the sawdust was weighed, stored, and a moisture determination was run.

The filtrate and aqueous wash were extracted with ether to remove neutrals. The filtrate was then acidified with dilute sulfuric acid and again extracted with ether. The aqueous solution was centrifuged and the clear solution was decanted away from the solid. The solid was covered with acetone and filtered. The acetone solution was bottled as was the solid after being air dried.

The clear aqueous solution was passed through a column packed with Amberlite IR-120 cation exchange resin. The resin was regenerated with hydrochloric acid and the regenerant effluent was saved. The resin was then washed with a solution of 4% sodium hydroxide and the eluate was saved. The original effluent of the filtrate was then passed through another column packed with Amberlite IRA-4B and the effluent was concentrated and bottled.

The results of the caustic hydrolyses follow.

CAUSTIC HYDROLYSIS OF ASPEN SAWDUST

FRACTION	WHOLE SAWDUST					75% PROPANOL EXTRACTED SAWDUST				
	Code No.	Total	Solids	MeO		Code No.	Total Solid	MeO		
		g./100g.	g.	%	g.		g./100g.	g.	%	g.
Sawdust Used	1392-38-A		100			1392-41-A	100			
Sawdust Recovered	1359-95-C		63.2			1359-105-A	73			
Ether Ext. of Caustic Sol.	1359-94-A	0.1	.002			1359-105-B	5.0 ²	0.01		
Ether Ext. of Acid Sol.(a)	1359-95-B	3.4	2.28			1359-106-A	1.53	1.03		
Acid-Eth-Ether Wash	1359-99-B*	6.2	3.35			1359-107-B	1.0	2.15		
HCl Regenerant	1359-97-B	2.6	1.64							
NaOH Wash of IR-120	1359-98-A	22.66	22.4							
Ion Free Aqueous Sol. From (a)	1259-99-A	4.39	18.4	1.36	0.25	1359-107-H	9.24	28.7	1.86	0.53
Acetone Sol. of Solid	1259-100-B	1.7	1.43			1359-107-D	14.4	5.0		
Solid	1359-100-A		6.5			1359-107-C	3.5			

*Wash consisted of dilute sulfuric acid followed with ethanol and ether.

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All the fractions were chromatographed. The results of these chromatograms follow.

1359-94-A Showed nothing with any of our phenol sprays.

1359-95-B Developed with BuOH-2% aq. amm.

Benzidine Rf- 0.00; 0.03; 0.10; 0.35; 0.49; 0.82.

Mäule Rf 0.03 0.35

Developed with pyridine-BuOH-water (3:10:3)

Benzidine Rf- 0.12; 0.18; 0.32; 0.45; 0.52; 0.56; 0.82

Mäule Rf 0.45 0.82

By comparison it was found that:

BuOH Rf 0.35 is syringaldehyde.

BuOH Rf 0.49 is vanillin

Py. Rf 0.45 is syringic acid

Py. Rf 0.52 is vanillic acid

Py. Rf 0.56 is p-hydroxybenzoic acid.

1359-97-B Developed with BuOH-2% aq. amm.

Benzidine Rf 0.00; 0.08; very heavy salt spot. No Mäule.

Developed with 10:3:3

Benzidine Rf 0.00; 0.64 very heavy salt spot and no Mäule.

1359-98-A Developed with BuOH 2% aq. amm.

Benzidine Rf 0.00; very heavy salt spot - no Mäule.

Developed with 10:3:3

Benzidine Rf 0.00; 0.07; very heavy salt spot - no Mäule.

1359-99-B Developed with BuOH 2% aq. amm.

Benzidine Rf 0.00; 0.03; 0.08; no Mäule.

Developed with 10:3:3

Benzidine Rf 0.00; 0.03; 0.77; no Mäule.

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1359-99-A Developed with BuOH 2% aq. amn.

Benzidine and Mäule Rf 0.00

Developed with 10:3:3

Benzidine and Mäule Rf 0.00

1359-100-B Developed with BuOH 2% aq. amn.

Benzidine and Mäule Rf 0.00; 0.03

Developed with 10:3:3

Benzidine and Mäule Rf 0.00; 0.08

1359-100-A Rf 0.00 in both developers - no Mäule.

1359-105-B Showed nothing with any of phenol sprays.

1359-106-A Developed with BuOH-2% aq. amn.

Benzidine Rf 0.00; 0.05; 0.10; 0.31; 0.47; 0.71; 0.82

Mäule Rf 0.00; 0.05; 0.32 0.71

Developed with 10:3:3

Benzidine Rf 0.45; 0.52; 0.56; 0.82

Mäule Rf 0.45 0.82

By comparison, this fraction contains the five compounds

listed for fraction 1359-95-B.

1359-107-A Developed with BuOH-2% aq. amn.

Benzidine Rf 0.00; 0.03; 0.12

Mäule Rf 0.00

Developed with 10:3:3

Benzidine Rf 0.00; 0.05

Mäule Rf 0.00

1359-107-B Developed with BuOH-2% aq. amn.

Benzidine Rf 0.00; 0.03; 0.12

Mäule Rf 0.00

Developed with 10:3:3

Benzidine Rf 0.00; 0.05; 0.60

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Måule Rf 0.00

1359-107-C Developed with BuOH-2% aq. amm.

Benzidine Rf 0.00; 0.03

Måule Rf 0.00

Developed with 10:3:3

Benzidine Rf 0.00; 0.05

Måule Rf 0.00

1359-107-D Developed with BuOH-2% aq. amm.

Benzidine Rf 0.00; traces of 0.10; 0.31; 0.47

Måule Rf 0.00; 0.31

Developed with 10:3:3

Benzidine Rf 0.00; traces of 0.56; 0.82

Måule Rf 0.00 0.82

A method of quantitatively determining the amounts of materials by ultra violet density readings was used to determine the concentrations of syringaldehyde, vanillin, syringic acid, vanillic acid and p-hydroxybenzoic acid.

The technique used is as follows:

For the aldehydes a known sample is streaked on Whatman No.1 paper and developed with n-butyl ether saturated with water.

For the acids a known sample is streaked on the paper and developed with butanol saturated with a buffer solution of pH- 6.6.

A single spot on the same sheet with the sample is sprayed to locate the desired materials. The area of the paper containing the sample is then cut and eluted in a micro Soxhlet with ethanol. The ethanol extract is treated with 5cc. of 0.2% potassium hydroxide in ethanol

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and diluted to 50cc. This sample is then read on the ultra violet spectrophotometer at a specified wave length. This density reading can then be converted into mg/liter by multiplying with a conversion factor determined from the graph plotting densities against concentrations.

Vanillin read at wave length	352.	$D \times 5.33 = \text{mg/liter}$
Syringaldehyde	368	6.76
Syringic acid	300	16.40
Vanillic acid	298	13.10
p-Hydroxybenzoic acid	278	8.30

Both ether extracts of the acidified solutions were spotted and concentrations of these materials were determined.

	1359-95-B		1359-106-A	
	mg. in sample, %		mg. in sample, %	
Vanillin	61.32	2.7	29.60	2.9
Syringaldehyde	96.60	4.2	57.35	5.5
Syringic acid	54.60	2.4	15.70	1.5
Vanillic acid	87.36	3.8	23.12	2.2
p-Hydroxybenzoic acid.	840.00	36.8	555.00	53.8

Acid hydrolysis of Aspen native lignin.

One gram of "Aspen native lignin" (IPC File no. 123419, notebook 103A679) was suspended in a solution of 5 grams sulfuric acid in 100cc. of water. The mixture was boiled under reflux for four hours. When cool, the mixture was extracted with ether. The ether was concentrated to a small volume and stored, 1359-118-A.

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The aqueous solution was decanted away from an insoluble solid. The insoluble solid was covered with ethanol, filtered, and the ethanol was concentrated to a small volume, 1359-118-B. The solid when dried was stored, 1359-118-C.

The aqueous solution was neutralized with barium carbonate, filtered, washed and concentrated to a small volume, 1359-118-D.

The four fractions were chromatographed with the following results:

1359-118-A and 1359-118-B were the same.

BuOH 2% aq. amm. Rf 0.10

The material was checked against authentic p-Hydroxybenzoic acid in butanol and also in 10:3:3 and matched.

1359-118-C Developed in BuOH-2% aq. amm.

Benzidine Rf 0.00; 0.02; 0.05

FeCl₃ Rf 0.00; 0.05

M&ale & 2,4 Rf 0.00

1359-118-D Developed in BuOH 2% aq. amm.

Benzidine Rf 0.00; 0.02

FeCl₃ Rf 0.00

No sugars were present in any of the fractions.

dlb/jw

PROJECT REPORT FORM

Copies to: Files Notebook 1633:
Dr. Pearl (2) pages 11-36,
Reading Copy 38-57,
78-106,
112-113,
115-128,
135, 137-142,
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Notebook 1739:
pages 11 and 15.

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NOTE BOOK 1633 and 1739
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SIGNED *Irwin A. Pearl*
Irwin A. Pearl

STUDIES ON GLUCOSIDES OF ASPEN BARK

In continuing our studies on the chemistry of the extractives of aspenwood it became of interest to study the extractives of aspen bark. This interest was occasioned by the unusual results obtained when the products of alkaline hydrolysis of aspen bark were compared with similar products from the alkaline hydrolysis of various portions of an aspenwood log (See Project 809-13, Report No. 25). It was desired to study the nature of aspen bark components as they occurred in the natural state instead of after alkaline hydrolysis. The present report describes experiments on the fractionation of several aspen barks and on the isolation and characterization of several glucosides from the water-soluble portion of the extractives. Work reported includes some experiments performed by Dr. Stephen F. Darling this past summer on the characterization of some of the glucosides.

For the first experiments three bags of mixed aspen bark from the mill pond of Green Bay Pulp and Paper Company were obtained on May 10, 1957 from Mr. Bill Nelson. This bark was of unknown origin and could be composed of one or more of the following woods employed by this mill: quaking aspen (Populus tremuloides), big tooth aspen (P. grandidentata),

or balsam poplar (P. *tacamahaca*). The brown bark was extracted with 95% ethanol, and the brown extract was concentrated to approximately 20% solids. This ethanol extract was referred to as mill run aspen bark extract.

A short while later (May 24, 1957) a sample of bark from known Populus *tremuloides* was obtained from Miss Barbara Reeder. Part of this green bark was air dried and Wiley milled. The bark dust was further air dried and then stored in a polyethylene bag. The rest of the bark was extracted with 95% ethanol and the extract was concentrated to approximately 20% solids. A complete report on the extraction of these two bark samples and on the composition of the extracts as determined by direct paper chromatography is found in Report No. 9 (Don Beyer).

The first experiment on the Breakdown of aspen bark extractives was performed on the mill run extract. A sample weighing 531 grams and containing 104 grams of solids was evaporated in a rotating evaporator below 30°. The residue obtained in this manner weighed 123 grams. This residue was covered with 500 ml. of petroleum ether (b.r. 60-110°) and allowed to stand for 24 hours with occasional shaking. The petroleum ether was decanted and evaporated in a rotating evaporator to give 1.8 grams of dark green residue. The residue was covered with a little water and evaporated under reduced pressure on a rotating evaporator to remove all petroleum ether. The residue was now treated with two liters of water, agitated vigorously for several minutes, and allowed to stand at room temperature overnight. The mixture was mixed with Celite and filtered through a Celite pad, and the precipitate was washed with water and air dried. The dried precipitate was stirred with ethanol, and the ethanol was filtered and concentrated. Nothing further was done with the ethanol extract.

The still turbid aqueous solution was treated with an excess of saturated basic lead acetate and filtered. The clear yellow filtrate was saturated with hydrogen sulfide, boiled to remove dissolved hydrogen sulfide, and filtered. The clear colorless filtrate was evaporated to dryness in a rotating evaporator to yield 53.7 grams of very viscous syrup which crystallized on long standing. The entire mixture was chromatographed on paper and developed in butanol-2% aqueous ammonia and in 10:3:3 butanol-pyridine-water developers. The papers were examined under ultraviolet light for fluorescence and sprayed with bis-diazotized benzidine and Maule test reagents. Results are given in Table I.

TABLE I

PAPER CHROMATOGRAPHY OF WATER EXTRACT OF
MILL RUN ASPEN BARK ETHANOL EXTRACTIVES
(R_f 's noted by bis-diazotized benzidine)

BuOH-NH ₃		BuOH-Pyr-H ₂ O	
R_f		R_f	
0.00 Maule		0.00 Maule	
0.07	+	0.02	+
0.20	+	0.07	+
0.41	+	0.40	+
0.49	-	0.57	+
0.55		0.67	
0.69		0.77	-
0.80	-		

(a) No spot with bis-diazotized benzidine

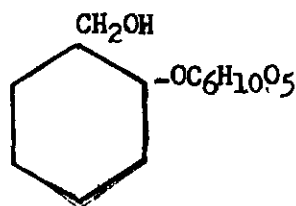
+ = white fluorescence under ultraviolet light.

- = negative fluorescence under ultraviolet light.

Thus it is apparent that this aqueous mixture contains a great number of compounds, many of which are phenolic in nature. Apparently, only the material with R_f 0.00 in both solvent systems contains the syringyl nucleus.

The entire mixture containing crystals was diluted with 95% ethanol and filtered. The white crystals were washed with 97% ethanol and air dried. The yield amounted to 3.75 grams of colorless crystals melting at 188-192°. Recrystallization from dilute ethanol raised the melting point to 194-197°. The crystals were very bitter in taste. Paper chromatography indicated no spot with fluorescence or with bis-diazotized benzidine, 2,4-dinitrophenylhydrazine, ferric chloride or Maule spray reagents. Permanganate-periodate spray indicated a spot with R_f 0.52 in the butanol-ammonia developer and R_f 0.55 in the butanol-pyridine-water developer. The specific rotation was $[\alpha]_D^{25} -61.2^\circ$. The bitter taste, melting point, and specific rotation agreed with published properties of salicin. A mixed melting point with authentic salicin proved the unknown material to be salicin.

The unknown material was acetylated with acetic anhydride and pyridine to give salicin pentaacetate melting at 126-127° and not depressing a mixed melting point with authentic salicin pentaacetate.



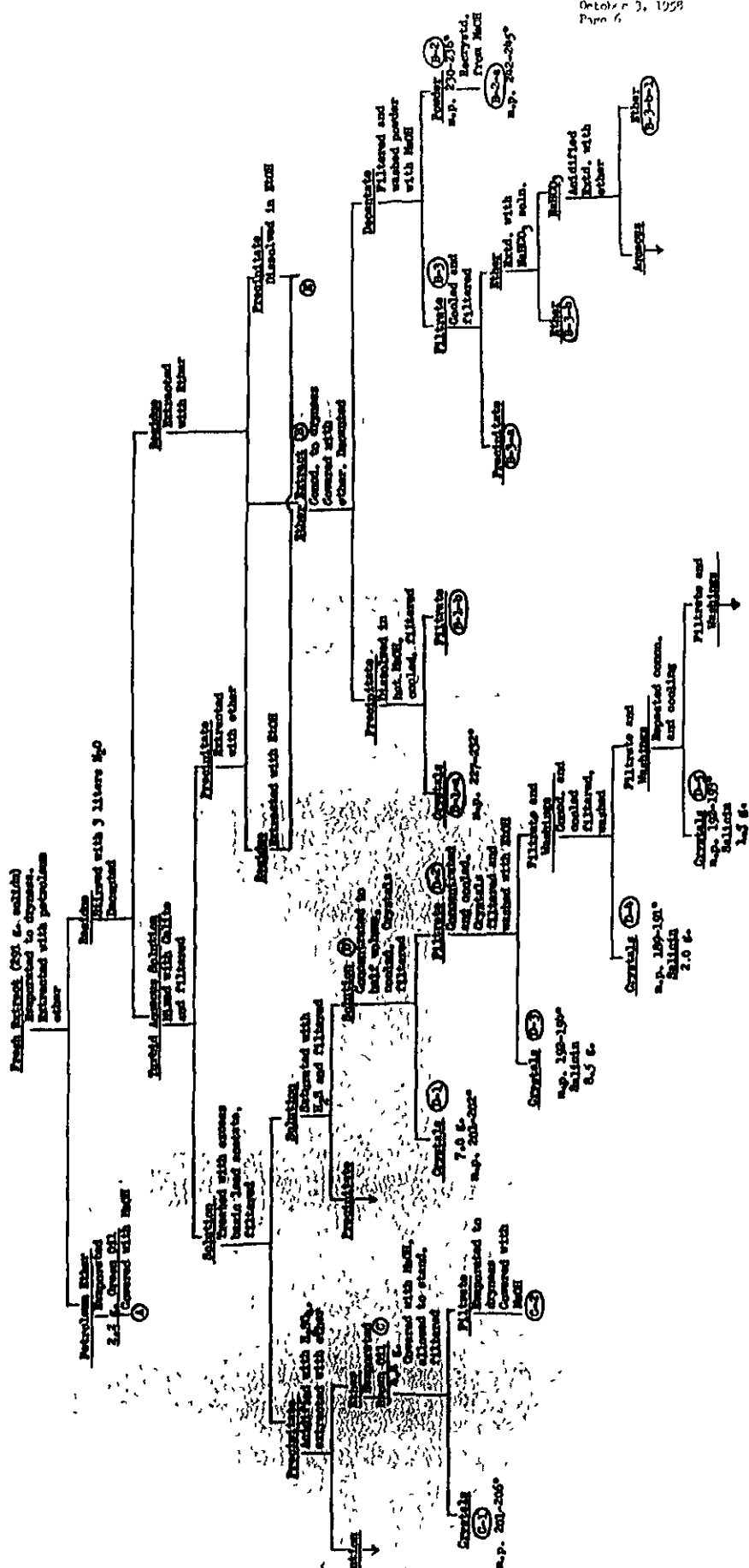
Salicin

In the next experiment the ethanol extractives of Populus tremuloides bark were employed. The concentrated ethanolic solution which had been standing at room temperature for seven days was shaken thoroughly and measured into a two-liter flask. The sample employed weighed 1093 grams and contained 291 grams of solids. The extract was evaporated to dryness in a rotating evaporator below 25°. The residue foamed when all solvent was removed. The dried extractives were processed in a manner similar to that described above. The complete processing is pictured in the flow sheet of Figure 1 in which all fractions are identified.

From the flow sheet of Figure 1 it is seen that the purified aqueous extract of the ethanol extractives of fresh aspen bark yielded a considerable amount of crystalline material (D-1) melting at 201-202° which was quite different from salicin in water solubility, melting point, and taste (this material was not bitter). This material was submitted to investigation in an attempt to determine its identity.

Several recrystallizations from dilute methanol raised the melting point to 205-206°. These crystals had optical activity, showing a specific rotation $[\alpha]_D^{31} +15.9^\circ (c = 2 \text{ in pyridine})$. Furthermore there appeared to be some evidence of mutarotation because the rotation changed from an initial value of +10.6° to the final value of +15.9° in 24 hours.

SALIENT TOPIC INFORMATION AND RELEVANT FACTS



One gram of fraction D-1 was covered with 150 ml. of 1% sodium hydroxide, and the mixture warmed to 50° and allowed to stand at room temperature overnight. The clear yellow solution was neutralized with dilute sulfuric acid and concentrated to about one-half volume in a rotating evaporator. Shiny crystals separated from solution. These were filtered and recrystallized from dilute ethanol to give colorless platelets melting at 119-120°. Analysis indicated benzoic acid, and a mixed melting point with benzoic acid was not depressed. The infrared absorption spectrum was identical with that of authentic benzoic acid.

Calculated for benzoic acid, $C_7H_6O_2$: C, 68.84; H, 4.95.

Found (Huffman): C, 68.87; H, 4.94.

The aqueous filtrate was concentrated further, and the precipitate was filtered and recrystallized from ethanol to give white crystals of salicin melting at 190-191° and not depressing a mixed melting point with authentic salicin. Thus, the unknown material appeared to be a benzoate of salicin. Analysis indicated that the compound was a monobenzoate of salicin.

Calculated for salicin monobenzoate, $C_{20}H_{22}O_8$: C, 61.53; H, 5.68.

Found (Huffman): C, 61.44; H, 5.66.

The compound was acetylated with acetic anhydride in pyridine and the product was recrystallized to give colorless crystals of the tetraacetate of salicin monobenzoate melting at 114-115°, and specific rotation $[\alpha]_D^{24} +33.9^\circ$ (c = 2.5 in chloroform)

Calculated for salicin monobenzoate tetraacetate, $C_{28}H_{30}O_{12}$:

C, 60.21; H, 5.41 Found (Huffman): C, 60.25; H, 5.36.

The analysis of the acetate confirms the monobenzoate of salicin composition for the unknown glucoside.

A review of the literature indicated that both salicin and a benzoate of salicin had been isolated from aspens, but the properties of the reported benzoate were somewhat different from those of our isolated product. Furthermore, it was noted that portions of the prior art in this field were somewhat hazy and needed clarification. At this point a complete analysis of the literature on the glucosides of aspens and the related willows was made, and is reported herewith. It was found that the literature of the genus Populus is intimately linked with that of the genus Salix, both genera comprising the Salix family. Therefore the literature analysis covers the glucosides of both genera.

The glucoside salicin was discovered in the bark of Salix helix in 1830 by Leroux (1) who noted the antipyretic action of the material. In the same year Braconnot (2) showed that salicin also occurs in European quaking aspen, Populus tremula, along with a new substance which he named "populin". Quoting from Braconnot's paper entitled, "Chemical Examination of Aspen Bark", he says, "Finding myself in a village where many fevers of various sorts were prevalent, and knowing that most of the villagers were too poor to obtain outside help, I sought if it were possible to find a substitute for quinine in some of the local plants." Going further Braconnot said, "It occurred to me that the bark of Populus tremuloides (American quaking aspen) is employed in the United States with much success in combating fever and the perfect counterpart in the forests of France of this American species is our Populus tremula (European quaking aspen)."

Braconnot does not say how he obtained the above information about American quaking aspen but it could be inferred that the early American pioneers learned about aspen bark extract from the Indians who used such bark and root infusions in medicine. In this manner the information might have been passed on to Europe.

In any case Braconnot prescribed a water extract of the bark of European quaking aspen to patients suffering from fever and much to his delight the fevers subsided. This prompted Braconnot to investigate the composition of the bark extract and lead to his discovery of the presence of salicin and the new glucoside, populin, which he demonstrated to be a benzoate of salicin.

Following the steps of these early French investigators, Herberger (3), found both salicin and populin in the leaves of the white poplar, Populus alba.

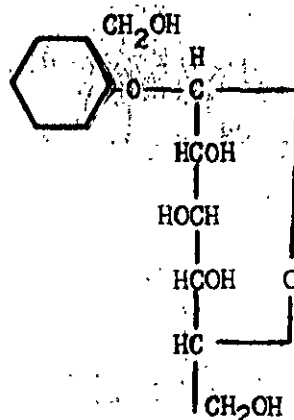
The science of organic chemistry was too much of an infant to afford these men with any means of arriving at the structural formulas for either of these compounds. Indeed, such a thing as a structural formula had not yet dawned on the scientific age. The determination of the empirical and partial structural formulas was first accomplished by Piria and Schiff and brought to its ultimate completion by Irvine and Rose and Richtmyer and Yeakel many years later.

Piria (4) began these structural proofs by showing that salicin could be hydrolyzed with dilute acid or enzymatically to yield saligenin and glucose. He proved that the phenolic hydroxyl in saligenin was involved in the glucosidic linkage because salicin could be oxidized to helicin,

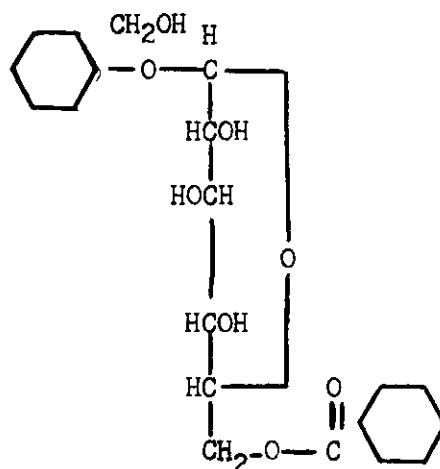
salicylaldehyde glucoside. He even showed the relationship between salicin and populin by demonstrating that populin yielded salicin and benzoic acid when saponified with barium hydroxide solution.

Later Schiff (5) showed that the benzoyl group in populin must be attached to the glucose because populin can be oxidized to benzoyl helicin, a substance he made by benzoylating helicin. Schiff also made what he believed to be synthetic populin by benzoylating salicin. He compared his synthetic populin with a sample of natural populin that came from Piria's laboratory in Turin. The trouble is that he based the identity of these two substances on such properties as taste, solubility, and action with concentrated sulfuric acid, properties we now know to be borne by similarly related and constituted substances. Dobbin and White (6) who improved Schiff's method for making synthetic populin continued this uncertainty by the statement that their synthetic populin was identical in all its properties with those of populin isolated from natural sources.

Thus the matter lay for over thirty years until Purdie and his school developed the next tool, methylation with silveroxide and methyl iodide, as a means of structure determination in the sugar field. In 1906 Irvine and Rose (7) completed the determination of the structure of salicin by making pentamethyl salicin from which they obtained by hydrolysis 2,3,4,6-tetramethylglucose. This last fact together with the fact that emulsin hydrolyzes salicin proves it to be salicylalcohol β -glucopyranoside.

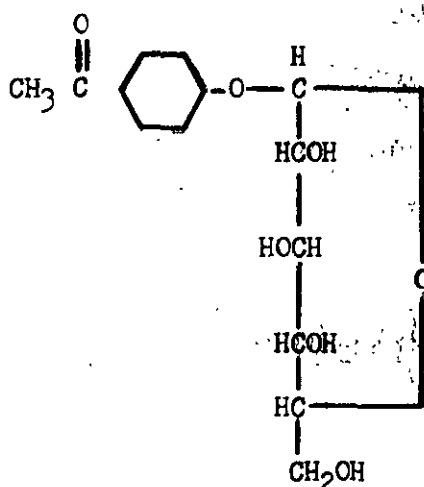


It remained for Richtmyer and Yeakel (8) to put the finishing touches on the proof of the structure of populin in 1934, that is, except for the proof that synthetic populin and populin from natural sources are identical. They did their work with synthetic populin made by an improvement in the method of Dobbins and White (6) and, therefore, were not dependent on limited amounts of material isolated from natural sources. These workers methylated synthetic populin by Furdie's method and obtained crystalline tetramethyl populin. Debenzoylation of this compound lead to tetramethylsalicin. Dilute acid hydrolysis of this tetramethylsalicin yielded a trimethylglucose which they proved to be 2,3,4-trimethylglucose by conversion into the known crystalline methyl-2,3,4-trimethylglucoside. These facts leave no doubt that synthetic populin is 6-benzoylsalicin. The only recorded reproducible physical properties for pure populin are those for synthetic populin (8)--melting point, 179° and $[\alpha]_D^{20} -2.0^\circ$ (c = 5 in pyridine).



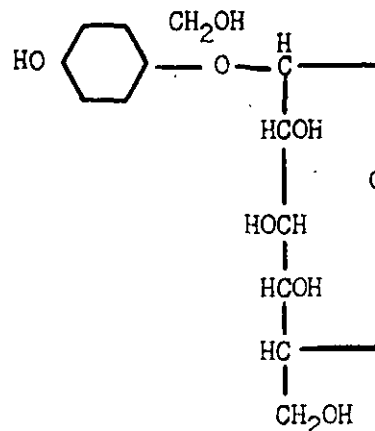
Populin

Picein, the third salix glucoside, was discovered by Tanret (9) in 1894 originally in Picea excelsa LK., whence the name. Later Jowett (10) reported the presence of a glucoside in the bark of a Salix species, probably discolor, which he called salinigrin. Johannson (11) and Jacoby (12) also reported a glucoside from Salix cinerea L. which they called salicinerin while Bridel, Charaux, and Rabate (13) reported still another glucoside from Ameliander vulgaris Moench in 1928 which they called ameliaroside. In 1929 Bridel and Rabate (14) finally showed that their ameliaroside, Jowett's salinigrin, and Johannson and Jacoby's salicinerin were one and the same thing and identical to picein. In the meantime Mauthner (15) showed that his synthetic glucoside made from p-hydroxyacetophenone and bromo-tetraacetylglucose was identical with the acetate of natural picein originally obtained by Tanret. Picein is therefore p-hydroxyacetophenone glucopyranoside.



Picein

In 1931 Wattiez (16) isolated a glucoside from Salix repens L. which he called salireposide. He was able to show that this new glycoside was a benzoylated glucoside by isolating benzoic acid and glucose from its hydrolysis products. The nature of the aglucone was proved in 1947 by Fujikawa and Akimasa (17) who debenzoylated the salireposide to give a crystalline debenzoylsalireposide melting at 172°. This was next methylated, oxidized with permanganate and hydrolyzed to give the known 2-hydroxy-5-methoxybenzoic acid. The debenzoylated salireposide must therefore be hydroxysalicin and salireposide is supposed to be hydroxypopulin. This latter assumption has not been confirmed to date (1958) because if salireposide is hydroxypopulin the position of the benzoyl group would have to be in the 6 place but this has not been demonstrated.



Debenzoylated salireposide

Two other salix glucosides have been reported in the literature but the structure of these is not known at present. They are salipurposide found in Salix purpurea L. by Charaux and Rabate (18) in 1931 and isosalipurposide reported by the same workers (19) in 1931 and 1933.

A summary of the glucosides and their properties heretofore reported from the barks of species in the Salix family is given in Table II.

TABLE II
HERETOFORE REPORTED SALIX GLUCOSIDES AND THEIR PROPERTIES

Name	m.p., °C.	Acetate m.p., °C.	Specific Rotation [α] _D
Salicin	200	131-132	-20.3° (pyr.) -63.6° (H ₂ O)
Populin	179		-2.0° (pyr.)
Picein	193-194	172-173	-87.8° (H ₂ O)
Salireposide	206 ^a	126.5	-36.8° (pyr.-acetone)
Salipurposide	227		-109.0° (95% EtOH)
Isosalipurposide	175		

(a) After first melting at 155-156° and resolidifying.

The distribution of the above mentioned glucosides in plant material has been the subject of a number of investigations. The most comprehensive survey of the existence of salicin and other glucosides in plant material appeared in 1932 by Thies and Wehmer in G. Klein (20) and by Wehmer (21). Here are listed thirty-two species of Salix and eight of Populus in which the presence of salicin has been reported. Populin appears to be much less widely distributed having been reported in only one species of Salix, namely purpurea, and in six species of Populus including alba, tremula, pyramidalis, tremuloides, nigra, and monilifera. Picein has been isolated from Salix discolor and Salix cinera while salireposide occurs only in Salix repens and Salix purpurea L. subspecies angustifolia Koidz according to Sakai, Tsurumi, Eno, and Mukai (22).

Unfortunately the presence of these glucosides has not always been demonstrated by actual isolation of the material but by indirect physical and chemical means. For example, Jowett and Potter (23), in their study of the occurrence of salicin and nicein in different willow and poplar barks apparently isolated salicin from only one species, S. rubra, although they reported salicin to be present in many other species based on an indirect proof. This latter consisted of quantitatively determining the glucose in the purified extract followed by hydrolysis with dilute sulfuric acid and determination of the glucose again. Any increase in the glucose content was attributed to the presence of salicin which on hydrolysis should form glucose and saligenin. They further tested for the saligenin by a colorimetric test for salicylic acid in part of the clarified bark extract that had been oxidized with sulfuric acid and potassium dichromate. This method while applicable to solutions containing only salicin and glucose would have little validity for a complex mixture like that of bark extract now known to contain sucrose and also now known to contain other possible glucosides which produce both glucose on hydrolysis and salicylic acid on oxidation.

In their study, "The Quantitative Determination of Salicin and Populin in Salix Species in 1937." A. Kuhn and G. Schafer (24) made two improvements in the method of quantitative estimation of salicin. Instead of determining the glucose on the clarified concentrate with Fehling's solution, they measured the optical activity of the clear solution. Then, to hydrolyze the salicin without hydrolyzing the sucrose, they introduced emulsin after which the optical activity was measured again. Knowing that emulsin will not hydrolyze sucrose, but will hydrolyze salicin to saligenin

and glucose, they felt confident the enhancement in the rotation of their solution after treatment with emulsin was a quantitative measure of salicin present. Even with these improvements it is still questionable in the light of our present knowledge whether the salicin and populin contents were actually being measured in every case.

In summing up it can be said that the presence of salicin in the barks of some Salix and Populus species has been demonstrated. The presence in the bark of some poplars and willows of populin is known with certainty in few cases. The existence of salicin, populin, and other glucosides in many species of Populus and Salix remains to be established by more reliable evidence than has heretofore been published.

Returning now to our investigations, we have found in the bark of Populus tremuloides a glucoside whose analysis and that of its acetate are the same as those of populin and populin tetraacetate and which, like populin, yields salicin and benzoic acid upon alkaline hydrolysis. However, the melting point and rotation are very different from those of synthetic populin. Therefore, the new glucoside, which we have termed "tremuloidin" is an isomer of synthetic populin.

Because, as noted earlier, the identity of synthetic populin with natural populin was based upon such properties as taste, solubility in water, etc., color with sulfuric acid and the like, it was possible that natural populin was identical with the material we had isolated as tremuloidin and not with synthetic populin. Thus, our problem was to determine the structure of tremuloidin and also to determine whether synthetic populin and natural populin were identical.

Authentic synthetic populin was prepared by the procedure of Richtmyer and Yeakel (8). A solution of 20 grams of salicin in 400 ml. of water was stirred mechanically in a one-liter beaker and treated dropwise during the course of one hour with 25 grams of benzoyl chloride always maintaining the reaction mixture alkaline to phenolphthalein by simultaneous addition of aqueous potassium hydroxide solution. Shortly after reaction began, a white granular precipitate began to separate. When addition was complete, the alkaline mixture was stirred a short while and filtered. The granular precipitate was washed thoroughly with water, transferred to a one-liter beaker, covered with 500 ml. of water, and heated to boiling. The boiling mixture containing some undissolved oily material was filtered hot. Upon cooling, the filtrate deposited white needles which were filtered to give 5.5 grams of product melting at 178-179°. Recrystallization from ethanol gave white needles with unchanged melting point and specific rotation $[\alpha]_D^{24} -2.0^\circ$ ($c = 5$ in pyridine). These are the same melting point and rotation recorded by Richtmyer and Yeakel for their synthetic populin.

A mixture of 3.5 grams of synthetic populin, 20 ml. of acetic anhydride and 30 ml. of pyridine was shaken and allowed to stand overnight. The mixture was stirred into 600 ml. of cold water, and the crystalline precipitate was filtered and recrystallized from ethanol to give silky white needles of synthetic populin tetraacetate melting at 126-126.5°. The specific rotation was $[\alpha]_D^{24} -6.0^\circ$ ($c = 3.74$ in chloroform).

Calculated for populin tetraacetate, $C_{28}H_{30}O_{12}$: C, 60.21; H, 5.41.

Found (Huffman): C, 60.19; H, 5.42.

Salicin was also acetylated in the same manner to give pentaacetyl salicin which was recrystallized from ethanol to give needles melting at 131-132° and having specific rotation $[\alpha]_D^{24} -18.0^\circ$ ($c = 4.35$ in chloroform). Brauns (25) reported a melting point of 130-132° and a specific rotation $[\alpha]_D^{20} -18.3^\circ$ ($c = 8$ in chloroform) for salicin pentaacetate.

PREPARATION OF PENTAMETHYLSALICIN--Salicin acetate was methylated by the general method of Haworth and Streight (22). Two grams of salicin pentaacetate was mixed with 40 ml. of acetone and introduced into a 250 ml. 3-neck flask equipped with silicone-sealed stirrer, thermometer, and two burets. With stirring 2 ml. of dimethyl sulfate and 3.2 ml. of 30% sodium hydroxide solution were added. Every 10 minutes 2 ml. of dimethyl sulfate and 3.2 ml. of sodium hydroxide solution were added until a total of 20 ml. of dimethyl sulfate and 32 ml. of sodium hydroxide solution had been added. After the last addition 15 ml. of water was added, and the temperature raised to remove the acetone. The aqueous residue was evaporated in the rotating evaporator to incipient turbidity and extracted with ether. The ether was dried and evaporated in the rotating evaporator to yield pentamethyl salicin as a colorless oil.

In order to obtain some authentic natural populin a batch of fresh leaves from an authentic *Populus alba* tree was extracted with hot water in accordance with Herberger (3), and the hot water extract was purified by means of basic lead acetate. The purified solution was freed from lead by means of hydrogen sulfide and concentrated somewhat. Some ash containing crystals which separated were filtered, and the clear filtrate evaporated further to yield crystals. These were recrystallized from water in the presence of decolorizing carbon to give colorless needles

melting at 179-180° and not depressing a mixed melting point with synthetic populin. The infrared absorption spectra of natural and synthetic populins were superimposable. In addition, the specific rotation of natural populin in pyridine was identical with that of synthetic populin. Thus, the identity of synthetic and natural populins was definitely established, and it became obvious that the product "tremuloidin" melting at 205-206° and with dextro rotation must be an isomer of populin which had not been reported heretofore. Furthermore, the monobenzoate substitution in tremuloidin must be at some position other than the 6-glucose position, which is the known benzoyl substitution of populin (8).

A controlled periodate oxidation of tremuloidin (see Project Report 15 by J. W. Green) indicated that only one mole of periodate was consumed, and no acidity was developed. These data were typical for compounds containing only two adjacent hydroxyl groups. Of the possible monobenzoates of salicin other than 6-glucose substitution, only monobenzoyl substitution at positions 2 or 4 on the glucose would satisfy these conditions. The anomalous dextro rotation of the acetate of tremuloidin seemed to indicate 2-glucose substitution rather than 4-glucose substitution. Pigman (26) showed that the acetates of several glucosides substituted in the 2-glucose position demonstrated this anomalous dextro rotation. However, Pigman's data were far from complete, and did not prove that any compound showing such dextro rotation must be substituted in the 2-glucose position.

The general methylation procedure employed by Richtmyer and Yeakel (8) for determining the location of benzoyl substitution in populin was employed for determining the location of benzoyl substitution in tremuloidin. For comparative purposes, experiments were carried out simultaneously on both tremuloidin and populin.

METHYLATION OF TREMULOIDIN--In a small flask fitted with a reflux condenser and silicone sealed stirrer was placed a mixture of 1.0 gram tremuloidin, 10 ml. methyl iodide, and 15 ml. absolute methanol. With stirring and boiling under reflux, 6.0 grams of freshly prepared silver oxide was added over a period of three hours in one gram lots. After the second addition, 5 ml. of acetone were added to completely dissolve all tremuloidin. The mixture was allowed to stand overnight and filtered. The silver oxide was washed thoroughly with acetone, and the combined filtrate and washings were evaporated to dryness in a rotating evaporator. The colorless syrup was dissolved in 10 ml. of methyl iodide and a few drops of methanol and methylated as before. The process was repeated three times making a total of four methylations. After the third methylation, the product was completely soluble in methyl iodide without the addition of methanol. The final product was obtained as 1.049 grams of clear colorless viscous syrup having a specific rotation $[\alpha]_D^{25} +6.56^\circ$ ($c = 4.2$ in chloroform). All attempts at crystallization of this syrup failed. Complete methylation of tremuloidin was demonstrated by the fact that upon hydrolysis with hydrochloric acid and paper chromatography of the hydrolyzate, this syrup yielded only a spot for a trimethyl glucose. The hydrolyzed syrup was spotted along with authentic 2,3-dimethylglucose, 2,3,6-trimethylglucose, and 2,3,4,6-tetramethylglucose and developed with 9:2:2 ethyl acetate-acetic acid-water. Spots were located by means of the silver spray of Trevelyan and coworkers (27). Thus, the colorless viscous syrup must be tetramethyltremuloidin.

METHYLATION OF POPULIN--Synthetic populin was methylated in the same manner and was obtained as glistening needles from ether-petroleum ether melting at 132-134° and having $[\alpha]_D^{25} -30.3^\circ$ ($c = 5.6$ in chloroform). Richtmyer and Yeakel (8) recorded melting point of 134-135° and specific rotation of $[\alpha]_D -31.7^\circ$ ($c = 5$ in chloroform) for tetramethylpopulin obtained in this manner. Hydrolysis with acid of tetramethylpopulin and chromatography as noted above gave only a spot for trimethylglucose.

DEBENZOYLATION OF TETRAMETHYLTREMULOIDIN--The general procedure employed by Richtmyer and Yeakel for the debenzoylation of tetramethylpopulin was used. A solution of 1.05 grams of tetramethyltremuloidin in 20 ml. of anhydrous methanol was treated with a solution of 0.1 grams of metallic sodium in 10 ml. of anhydrous methanol, and the mixture was boiled under reflux for 10 minutes, diluted with 30 ml. of water, and evaporated somewhat under a rotating evaporator to remove all methanol. The milky aqueous solution was extracted with ether, and the ether was washed with water, dried with sodium sulfate, and evaporated in the rotating evaporator. The residual colorless oil solidified after a short while. The solid was dissolved in a little anhydrous ether and filtered. The clear filtrate was treated with petroleum ether (b.p. 30-60°), and the resulting clear solution placed in the freezer. Colorless needles separated. These were filtered and recrystallized from ether-petroleum ether in the same manner to give 0.31 gram of colorless needles melting at 85-86°, and having the specific rotation $[\alpha]_D^{25} -39.1^\circ$ ($c = 1.2$ in chloroform). Analysis indicated that this compound was tetramethylsalicin.

Calculated for tetramethylsalicin, $C_{24}H_{26}O_7$: C, 59.63; H, 7.65; OCH_3 , 36.3. Found (Huffman): C, 59.64; H, 7.68; OCH_3 , 36.1

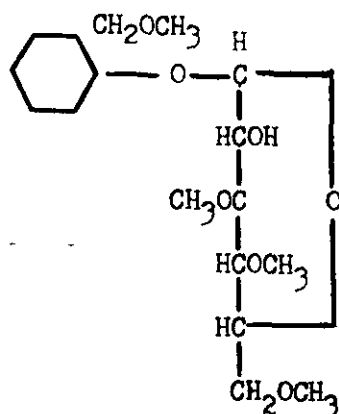
The aqueous layer remaining after the ether extraction above was acidified with hydrochloric acid and allowed to stand. The crystals which had deposited were filtered and recrystallized from water to give colorless crystals melting at 119-121° and not depressing the melting point of a mixture with authentic benzoic acid.

DEBENZOYLATION OF TETRAMETHYLPAPULIN--Tetramethylpapulin was debenzoylated with sodium methylate in methanol in the same manner to yield benzoic acid and a tetramethylsalicin melting at 136°. Richtmyer and Yeakel (8) recorded a melting point of 137-138° and a specific rotation $[\alpha]_D -46.4^\circ$ ($c = 3$ in chloroform) for *w*,2,3,4-tetramethylsalicin prepared in the same manner from tetramethylpapulin. Thus, the tetramethylsalicin obtained by debenzoylating tetramethyltremuloidin is entirely different from that obtained from tetramethylpapulin.

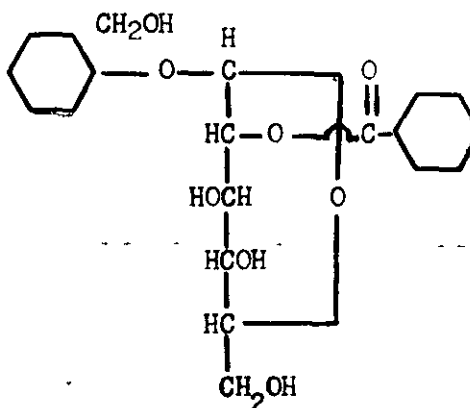
HYDROLYSIS OF TETRAMETHYLSALICIN FROM TREMULOIDIN--To further establish the structure of the tetramethylsalicin from tremuloidin, the position of the three methyl groups on the glucose portion of the molecule were determined by acid hydrolysis. For this purpose a mixture of 0.35 gram of the tetramethylsalicin melting at 85-86°, 4 ml. of methanol, and 6 ml. of 2N hydrochloric acid was heated on the steam bath under reflux for 2 hours. The mixture became milky after one hour and had deposited a reddish gum after two hours. Methanol was removed under reduced pressure in the rotating evaporator, and the residual aqueous mixture was filtered with the aid of a little Celite. The clear filtrate was treated with an excess of IR-4B ion exchange resin in the acetate form to remove chlorides and filtered. The resin was washed with an equal volume of water to give approximately 250 ml. of clear filtrate and washings. This clear aqueous

solution was concentrated to dryness in the rotating evaporator to leave a slightly yellow syrup. Paper chromatography of this oil in 9:2:2 ethyl acetate-acetic acid-water and spraying with para-anisidine indicated the presence only of a trimethylglucose and some phenolic aglucone material, with considerable distance between the two spots. The entire yellow syrup was dissolved in 2.5 ml. of methanol and streaked on four eight-inch wide Whatman 3M heavy papers. These papers were developed in the ethyl acetate-acetic acid-water developer. One-fourth inch strips were cut from each paper and sprayed with the para-anisidine to locate the band of trimethylglucose. The located bands were cut from the papers and eluted with methanol in a Soxhlet extractor. The methanol eluate was evaporated in a rotating evaporator to yield a colorless syrup. Paper chromatography indicated a trimethylglucose with the same R_f as 2,3,6-trimethylglucose in the ethyl acetate-acetic acid-water developer, but slightly different R_f in 10:3:3 butanol-pyridine-water developer. Furthermore, both chromatographic solvent indicated that the material was slightly impure. The crude syrup was purified by rechromatography on completely methanol-washed Whatman 3M paper and development in the ethyl acetate-acetic acid-water solvent. Elution of the indicated spots and evaporation of the methanol solvent as described above left a colorless syrup without any trace of impurity by chromatographic analysis. This syrup was seeded with a trace of authentic 3,4,6-trimethylglucose (3,4,6-tri-O-methyl-D-glucopyranoside kindly furnished by Dr. Nelson K. Richtmyer, National Institutes of Health, Bethesda, Maryland). After two weeks a considerable amount of crystals had separated in the syrup. The mixture was covered with a few ml. of purified diisopropyl ether and filtered. The crystals were recrystallized from two ml. of

diisopropyl ether to give colorless crystals melting at 97-98° and not depressing a mixed melting point with authentic 3,4,6-trimethylglucose. The infrared curves of the authentic 3,4,6-trimethylglucose and the trimethylglucose obtained by hydrolysis of tetramethyl salicin from tremuloidin were identical in every respect indicating that the tetramethylsalicin from tremuloidin is ω ,3,4,6-tetramethylsalicin and that tremuloidin is 2-benzoylsalicin.



ω ,3,4,6-Tetramethylsalicin



Tremuloidin
 (2-Benzoylsalicin)

FURTHER CHARACTERIZATION OF 3,4,6-TRIMETHYLGLUCOSE--While waiting for the syrupy trimethylglucose to crystallize it was further characterized by the periodate oxidation procedure of Lemieux and Bauer (28) who employed the method for separately identifying monomethylglucoses. One milligram each of the syrupy trimethylglucose, authentic 3,4,6-tri-O-methyl-D-glucopyranoside, and authentic 2,3,6-tri-O-methyl-D-glucopyranoside (kindly furnished by Dr. Fred Smith of the University of Minnesota, St. Paul, Minnesota) was placed in 13 x 75 mm. test tubes. To each tube in a beaker of ice and water was added 0.12 ml. of 0.5N sodium periodate solution.

The tubes were allowed to stand in the ice bath for one hour. They were then removed, treated with a few milligrams of ethylene glycol, and allowed to warm to room temperature and remain at this temperature for ten minutes. The latter procedure destroys the excess periodate. Each solution was spotted on Whatman No. 1 paper along with unoxidized trimethyl glucose and a known mixture of methylated glucoses. The chromatogram was developed in 5:1:4 butanol-ethanol-water, and the spots were located by means of an aniline-phthalic acid spray. The 2,3,6-trimethylglucose gave only one spot of unchanged trimethylglucose indicating no reaction in this oxidation procedure. The 3,4,6-trimethylglucose gave a strong spot much lower on the chromatogram than even the tetramethylglucose range plus a weak spot in the trimethylglucose range. This would seem to indicate strong cleavage presumably to a trimethylpentose. The unknown syrupy trimethylglucose gave spots identical in position and color with those from 3,4,6-trimethylglucose, again confirming the identity of these materials.

FURTHER ISOLATIONS OF GLUCOSIDIC MATERIAL

During the course of this investigation other attempts at isolating tremuloidin were made. The next experiment was made on the same concentrated ethanolic extract employed in the last isolation. This experiment was performed after the concentrated ethanolic extract had been standing at room temperature for 22 days. The last experiment was repeated, but the purified aqueous extract did not yield any tremuloidin before depositing the salicin. The experiment was repeated after the concentrated ethanolic solution had been standing for 74 days, and again, the concentrated purified aqueous extract yielded only salicin. Apparently, tremuloidin

extraction by cold water from the ethanol extractives of fresh Populus tremuloides bark is hindered in some manner by standing in ethanolic solution. Accordingly, the residue from the last cold water extraction was re-extracted with boiling water to determine whether tremuloidin was still in the extractives.

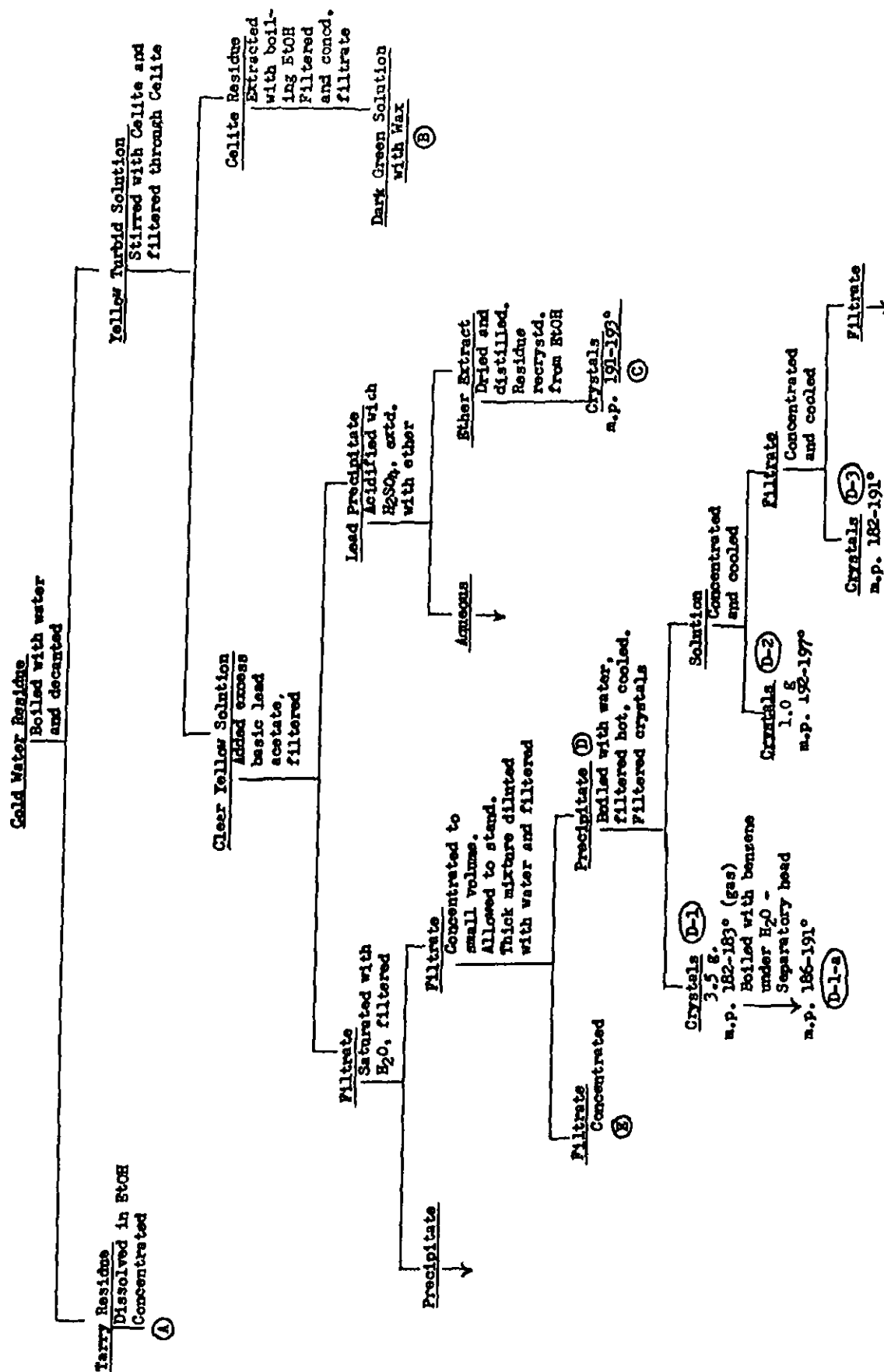
The cold water extracted residue from the last experiment was covered with one liter of hot water and boiled under reflux for one hour. The mixture was allowed to cool, and the turbid yellow aqueous mixture was decanted from the heavy oil. The hot water extraction was repeated on the heavy oil, and the combined turbid aqueous extracts were filtered through a Celite pad. The filtered aqueous extract was processed in accordance with the flow sheet of Figure 2 in which all fractions are identified.

From the flow sheet of Figure 2 it is apparent that pure tremuloidin did not separate from the hot water extract as it did in the original cold water extraction experiment.

The experiment of Figure 2 was repeated, but in this case, the lead purified solution was concentrated to a larger volume than that employed in the experiment of Figure 2. The processing of this lead purified solution is pictured in the flow sheet of Figure 3 in which fractions are again identified. Other fractions have same identity as those of Figure 2.

Figure 2

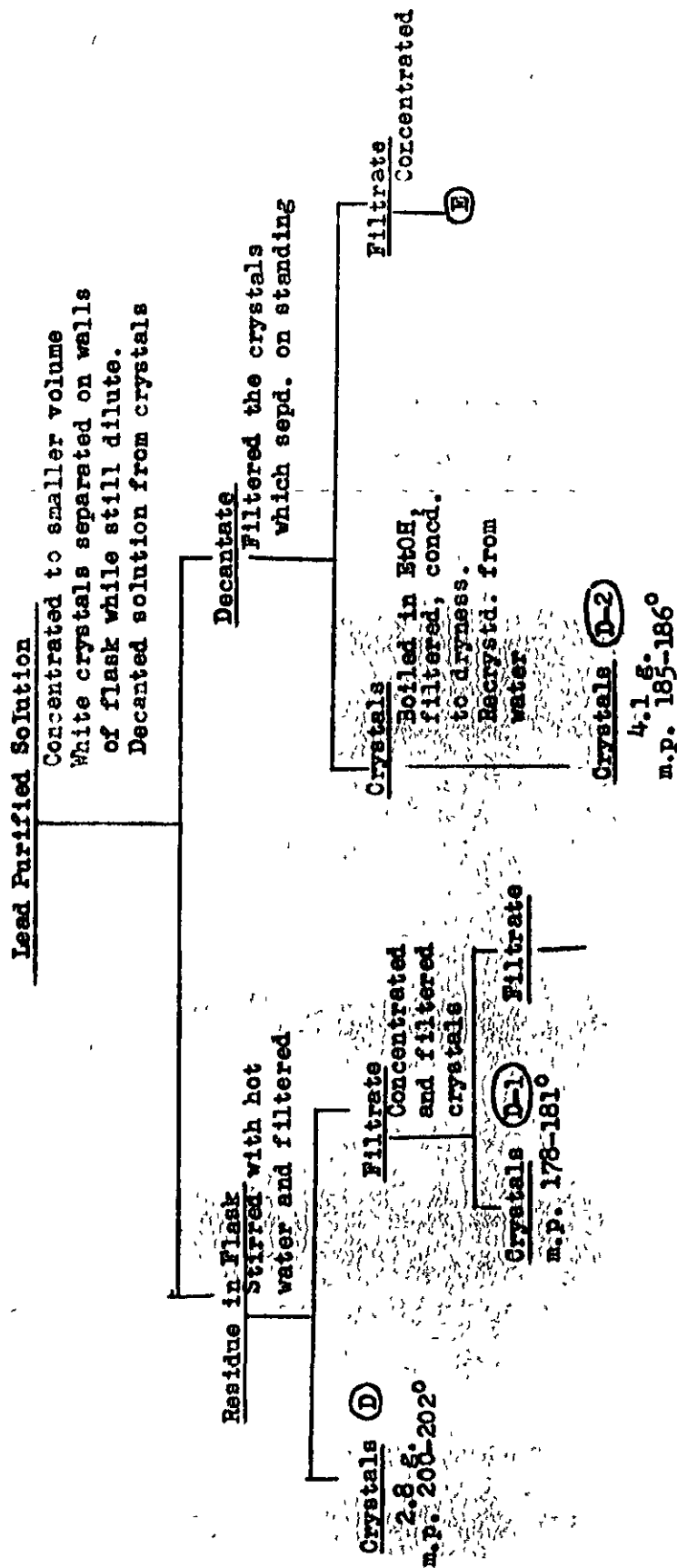
FRACTIONATION OF HOT WATER EXTRACTIVES OF FORULUS TREMULOIDES ETHANOL EXTRACTIVES



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Figure 3

FRACTIONATION OF LEAD PURIFIED AQUEOUS SOLUTION FROM
HOT WATER EXTRACTION OF ETHANOL EXTRACTIVES



In the next experiment the ethanolic extract was extracted directly with hot water without previous cold water extraction. Three thousand grams of the same concentrated fresh aspen bark extract employed in the last experiments after standing at room temperature for 200 days was evaporated to dryness in a rotary evaporator. The 800 grams of residue was covered with 2500 ml. of water and heated on the steam bath for 4 hours. After standing overnight, the aqueous layer was decanted. The oily layer was re-extracted with 2500 ml. of hot water in the same manner, and the turbid aqueous layers were combined, purified by means of basic lead acetate, and concentrated to about 2000 ml. Upon standing for several days crystal separated. These were filtered to yield 3.1 grams of white crystals melting at 151-153° with gas evolution. Recrystallization from water gave crystals melting at 153-156°, resolidifying, and melting at 199°.

The filtrate was concentrated somewhat and allowed to stand. A further crop of 3 grams of crystals melting at 199-202° was obtained.

RE-EXAMINATION OF DRIED FRESH ASPEN BARK--The anomalous results obtained with the concentrated ethanolic extract of fresh Populus tremuloides bark and the inability to duplicate the original isolation of tremuloidin when employing the extract after standing led to an investigation of the original bark which had been air dried and Wiley milled.

In a preliminary experiment 1086 grams of such air dried bark dust from the same bark employed in the original ethanolic extraction and containing 1000 grams of oven dried bark was stirred at room temperature with 7 liters of water for 2 hours, allowed to stand 2 hours, and filtered through a cotton cloth. The turbid filtrate was stirred with Celite and filtered through a Celite pad to give a clear brown solution. This solution was

purified by means of basic lead acetate, and the resulting colorless solution was concentrated in the rotary evaporator to 400 ml. volume and allowed to stand. The thick crystalline precipitate which separated was filtered and washed with a little cold water to give 6.2 grams of crystalline solid melting at 192-196°. Recrystallization from water raised the melting point to 204-205°, and the recrystallized product did not depress the melting point when mixed with authentic tremuloidin.

The bark dust after cold water extraction was stirred with 4 liters of water while heating to boiling for two hours. The mixture was allowed to cool overnight and was then filtered through a cloth. The turbid filtrate was purified as before and concentrated to yield 3.6 grams of crystals melting at 198-200°. Recrystallization by dissolving in hot methanol, filtering, diluting, and concentrating somewhat in the rotary evaporator yielded white crystals melting sharply at 207-208° and not depressing a mixed melting point with authentic tremuloidin.

The encouraging results obtained in the last experiment led to a repeat experiment on a larger amount of the air dried bark dust. The procedure employed was essentially similar to that employed before and is pictured in the flow sheet of Figure 4 in which all fractions are identified.

During the course of the investigation pictured in Figure 4 it was noted that ether extraction of the concentrated aqueous solution after separation of tremuloidin yielded additional tremuloidin or similar material. Accordingly, the residual aqueous solution [(G) of Figure 4] was diluted to one liter and extracted continuously in the air agitated liquid-liquid extractor (29). A flow sheet of this additional study is pictured in Figure 5 which identifies all fractions.

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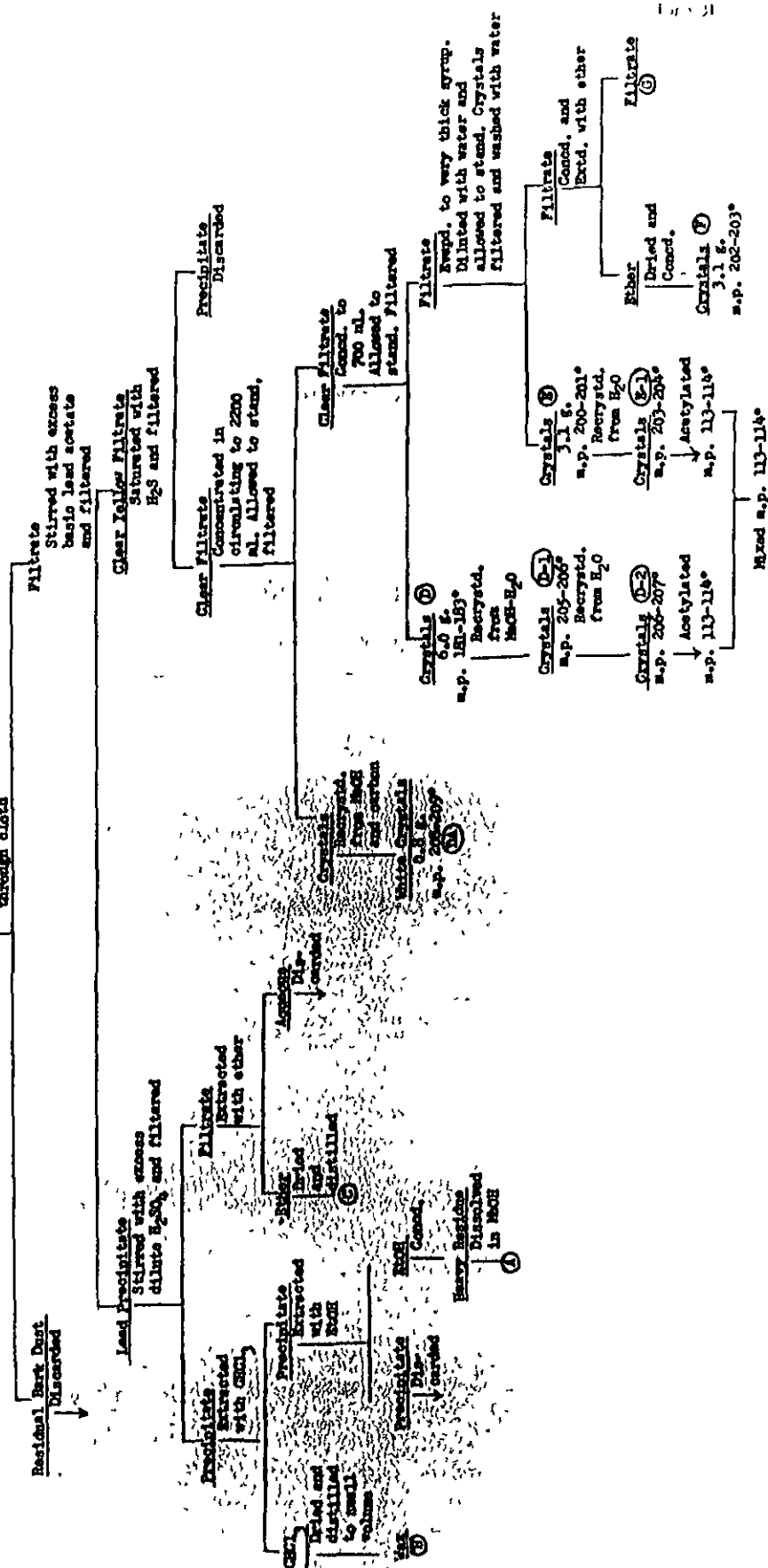
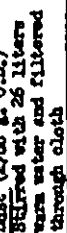
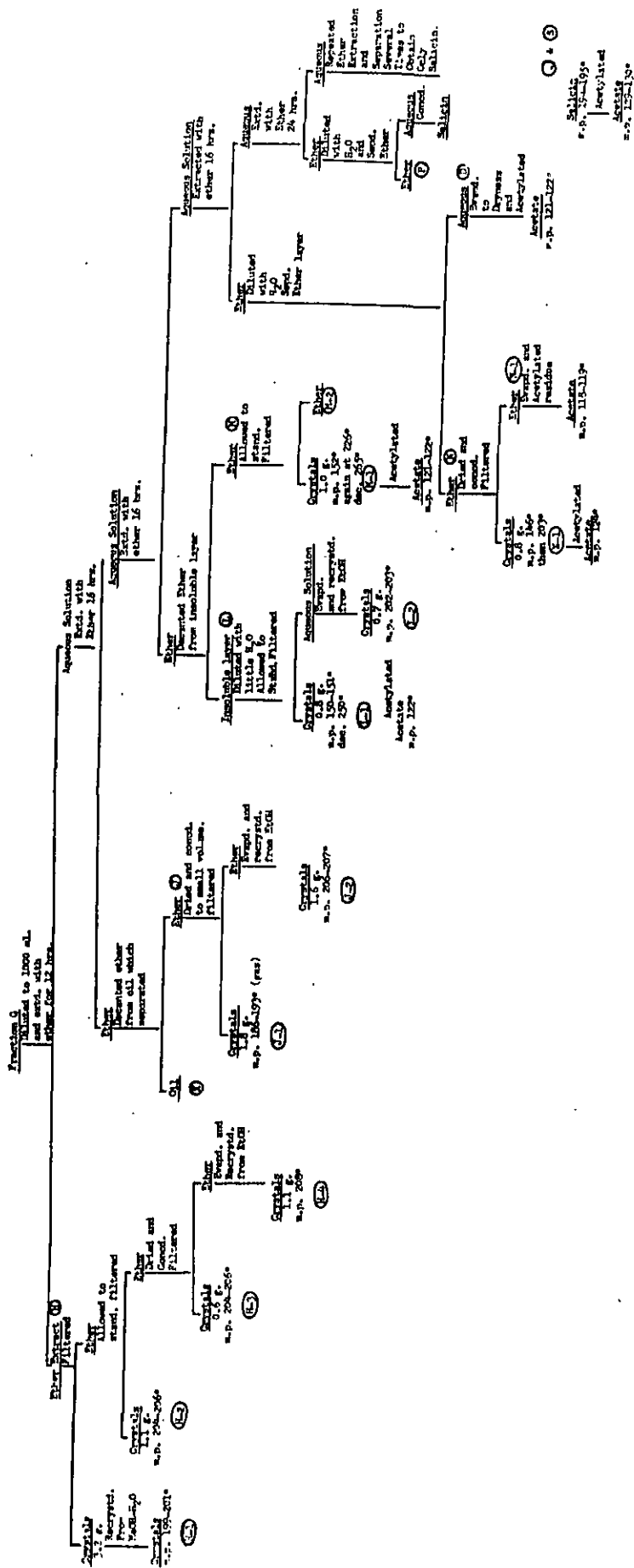


Figure 5.

FORSTER FRACTIONATION BY IRRADIATION OF FRACTION "C" OF FIGURE 4



The experiment of Figure 5 demonstrates that tremuloidin can be extracted preferentially from the concentrated aqueous solution. After most of the tremuloidin is removed, the ether extracts another material which melts about 150°, then solidifies and remelts about 202-204°. This material forms an acetate which melts at 121-122° when recrystallized from ethanol. Finally, the ether extracts salicin (acetate melting at 129-130°) from the aqueous solution.

CHROMATOGRAPHY OF GLUCOSIDES

The isolation of glucosidic materials with varying melting points from the aqueous extracts of Populus tremuloides indicated the presence of two or more compounds in most of the fractions. Separation and identification of components of the various fractions required methods other than solvent recrystallization. Paper chromatography offered a rapid method for qualitative separation and identification and was employed for these purposes. (Note; Some reference has already been made to paper chromatography in connection with the methylation studies noted earlier.)

It was found that the two developing systems that we had been employing in our carbohydrate studies served admirably for the separation of salicin, populin, and tremuloidin. These developing systems were 10:3:3 butanol-pyridine-water and 9:2:2 ethyl acetate-acetic acid-water. These developers will be referred to as BPW and EAW in this report. Location of the glucosides on developed chromatograms was obtained at first by use of the silver procedure reported by Trevelyan and coworkers (27) for the location of sugars. This spray was the only one of the several sugar sprays (periodate-permanganate, p-anisidine, and aniline-phthalic acid) tried which gave satisfactory results. The Trevelyan silver spray is employed

as follows: The chromatogram is thoroughly dried and then dinned in a tray containing 3% silver nitrate solution prepared by dissolving 3 grams of silver nitrate in 5 ml. of water and diluting with 95 ml. of pure acetone. The paper is allowed to dry after which it is uniformly sprayed with a 2% solution of sodium hydroxide in 95% ethanol. After standing at room temperature for a period of five to ten minutes to develop the spots, the paper is washed in 6N ammonium hydroxide to remove unreduced silver oxide. The paper is finally washed in water and dried. The sugars appear as dark brown spots against a light brown background.

NEW METHOD FOR DETECTING CARBOHYDRATE MATERIALS--The Trevelyan procedure was not satisfactory for the location of small amounts of glycosidic materials because the difference in color intensity of the glycoside spot and that of the background was not sufficiently great. Accordingly, a modification of the procedure was made in which a sodium thiosulfate wash was employed for removing unreduced silver oxide from the paper instead of the 6N ammonium hydroxide. This is the solution employed in photography for removal of unreduced silver compounds. In the modified procedure, the paper, after standing at room temperature to develop the spots, is bathed a few times in a strong solution of sodium thiosulfate (350 grams per liter), washed in water to remove excess thiosulfate and dried. In the modified procedure, the glycoside spots appear as almost black spots against a white background. Thus, much smaller amounts of glycosidic materials can be found. It is important in this modified procedure that all excess sodium thiosulfate be removed from the paper by washing with water. Otherwise, the spots will gradually fade over a period of a week or more. Hathaway and Seakins (30) have just published a procedure employing a 4% sodium thiosulfate wash for the Trevelyan procedure, but the two concentrations of thiosulfate have not been compared.

The three glucosides, salicin, populin and tremuloidin were easily recognized separately or in a mixture of all three when developed in either BPW or EAW systems and sprayed with the modified silver spray. R_f values at 25° for BPW were: salicin, 0.52; populin, 0.69; and tremuloidin, 0.77. R_f values for EAW were: salicin, 0.60; populin, 0.84; and tremuloidin, 0.85.

Because the presence of populin in the extractives of P. tremuloides bark had been reported in the past on the basis of indirect evidence (20,31) there was a possibility that the benzoate may actually have been tremuloidin. Accordingly, a number of impure fractions noted in Figures 1, 2, 3, and 4 were submitted to paper chromatography. Results are given in Table III.

TABLE III
CHROMATOGRAPHIC EXAMINATION OF IMPURE FRACTIONS

<u>Figure</u>	<u>Fraction</u>	<u>Comments</u>
1	D-1	Tremuloidin only.
1	D-4	Salicin only plus several sugars.
2	D-2	Tremuloidin with some salicin and a little sugar.
3	D	Tremuloidin only.
3	D-1	Equal parts of tremuloidin and populin.
3	D-2	About three parts tremuloidin to one part populin.
4	DA	Tremuloidin only.
4	D-1	Tremuloidin only.
4	D-2	Tremuloidin only.
4	E	Tremuloidin only.
4	F	Tremuloidin only.

Thus, it is apparent that populin actually exists in P. tremuloides extractives because it was found in substantial quantities by chromatography in the impure tremuloidin samples of the experiment of Figure 3. In order to obviate the possibility that populin may have been an artifact produced during the processing of the ethanol extractives a sample of fresh P. tremuloides bark was extracted directly with boiling water. Part of the water extract was purified by means of basic lead acetate. Both purified and unpurified aqueous extracts were concentrated under reduced pressure to syrups and chromatographed. Both syrups gave spots indicating the presence of salicin, tremuloidin, populin, four sugars in fair amount, and three sugars in small amount. In addition, the purified syrup gave spots for new as yet unidentified glucosides with BPW R_f 's 0.41 and 0.58.

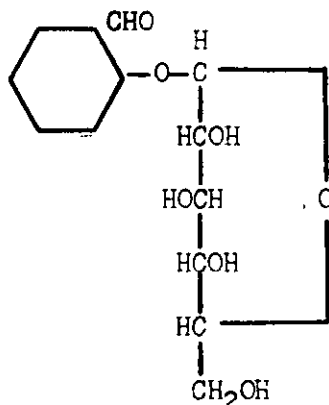
ISOLATION OF PURE POPULIN FROM P. TREMULOIDES---Fraction D-2 from the experiment of Figure 3 which was shown by paper chromatography to contain both tremuloidin and populin was submitted to column chromatography to determine whether these two glucosides could be separated on a preparative scale and to isolate some populin in crystalline form from P. tremuloides for comparison with authentic synthetic populin. A sample of Fraction D-2 (0.1 gram) was dissolved in EAW and absorbed on a dry-packed column of powdered cellulose (Whatman Standard Grade) 2 cm. in diameter and 15 cm. in height. The column was eluted with EAW, and the eluate was collected in 5 ml. fractions. The fractions were monitored by means of paper chromatography. The chromatograms indicated Fraction 1 contained only tremuloidin; 2, mostly tremuloidin with a little populin; 3, populin only; 4, populin only; 5, populin only; 6, trace of populin; 7 ff., blank.

Upon standing, Fraction 1 deposited crystals of pure tremuloidin melting at 209-210°. This melting point was the highest ever obtained for tremuloidin, and apparently, the chromatographic purification gave a purer product than could be obtained by recrystallization.

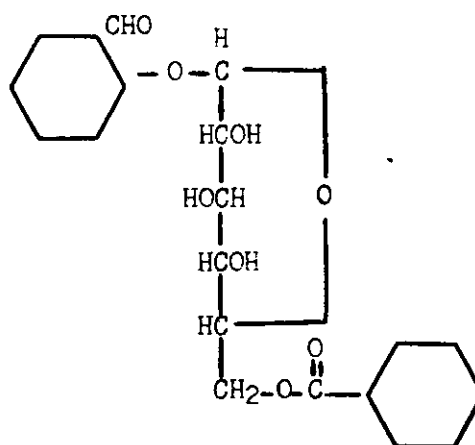
Fractions 4 and 5 were evaporated to dryness, and the residue was covered with water and filtered. The crystals melted at 179-180° and did not depress a mixed melting point with authentic synthetic populin.

SYNTHETIC STUDIES

In 1845 Piria (4a) reported the oxidation of salicin with nitric acid ($D = 1.16$) to yield helicin, and glucoside of salicylaldehyde. In 1855 Piria (4b) oxidized populin in the same manner with nitric acid ($D = 1.3$) to yield benzohelicin and in 1870 Schiff (5) demonstrated that benzohelicin prepared by nitric acid oxidation of populin was identical with that prepared by benzylation of synthetic helicin. The location of the benzoyl group in populin by Richtmyer and Yeakel (8) establishes the structure of Piria's and Schiff's "benzohelicin" as 6-benzohelicin.



Helicin



6-Benzohelicin

HELICIN--Several attempts to prepare helicin by treatment with nitric acid ($D = 1.16$) resulted only in the formation of nitrosalicylic acid. Schiff (5) noted that the nature of the nitric acid and the reaction conditions were critical for the oxidation of salicin, but even following his directions, no helicin was obtained. It is possible that our nitric acid is too pure for this purpose. Nothing further was done on this synthesis.

6-BENZOHELICIN--The procedure of Piria (4b) was employed. One gram of synthetic populin was placed in a 6.5 cm. Petri dish and covered with 11 ml. of nitric acid ($D = 1.3$). A microdrop of concentrated nitric acid was added, and, after a few minutes nitrous oxide fumes were evolved, and the populin went into solution. After five to ten minutes, small needles began to precipitate, and in a short while the entire mixture turned to a crystalline mass. The mixture was filtered, and the precipitate was washed with water. The diluted filtrate deposited more crystals which were filtered and added to the first batch. The crude product was recrystallized from water to yield fine colorless crystals of 6-benzohelicin melting at $179-180^\circ$, and with specific rotation $[\alpha]_D^{25} = 3.75^\circ$ ($c = 2.7$ in 80% acetone). A mixed melting point with the starting populin (m.p. $179-180^\circ$) was depressed considerably. Neither Piria nor Schiff recorded the melting point of 6-benzohelicin.

Calculated for 6-benzohelicin, $C_{20}H_{20}O_8$: C, 61.85; H, 5.19.

Found (Huffman): C, 61.77; H, 5.30.

6-BENZOHELICIN TRIACETATE--6-Benzohelicin was acetylated with acetic anhydride and pyridine at room temperature. After standing overnight,

the clear solution was poured with stirring into excess cold water. The precipitate was filtered, washed with water, and recrystallized from dilute ethanol to give colorless crystals of 6-benzohelicin triacetate melting at 125-126°.

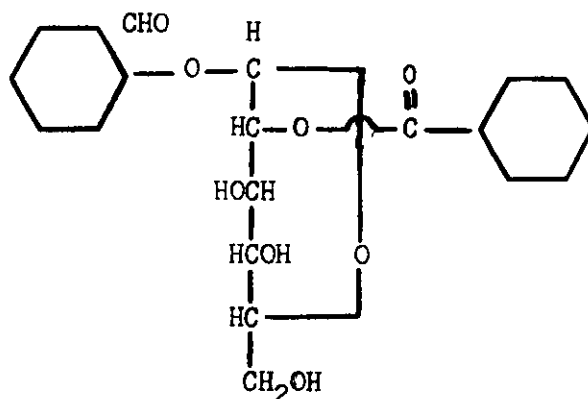
Calculated for 6-benzohelicin triacetate, $C_{26}H_{26}O_{11}$: C, 60.70; H, 5.09. Found (Huffman): C, 61.04; H, 5.16.

2-BENZOHELICIN--Tremuloidin (2-benzoylsalicin) was oxidized in the same manner with nitric acid ($D = 1.3$). One gram of tremuloidin was placed in the Petri dish and covered with the nitric acid. One microdrop of concentrated nitric acid was added. The tremuloidin did not dissolve completely as did the populin under the same conditions. After 20 minutes nitrous oxide began to be evolved, and the character of the crystals changed. After another 20 minutes, the completely changed crystals were filtered, washed with water, and recrystallized from water to give faintly yellow crystal of 2-benzohelicin melting at 213-214°. The product was dissolved in 80% acetone, treated with decolorizing carbon, and filtered. The colorless filtrate was evaporated in the rotating evaporator to remove the acetone, and the residual solution deposited colorless needles of 2-benzohelicin melting at 214-215°, and having a specific rotation $[\alpha]_D^{25} -25.9^\circ$ ($c = 3.7$ in 80% acetone).

Calculated for 2-benzohelicin, $C_{20}H_{20}O_8$: C, 61.85; H, 5.19.
Found (Huffman): C, 61.78; H, 5.26.

Both 6-benzohelicin and 2-benzohelicin were chromatographed on paper and developed with EAW. Both had identical R_f 's of 0.89. When sprayed with the modified silver spray, both spots appeared instantaneously whereas the parent populin and tremuloidin appeared only after standing at room temperature,

In addition the two benzohellicins gave spots with 2,4-dinitrophenylhydrazine, but the parent compounds did not. With this carbonyl reagent 6-benzohellicin gave an orange-yellow spot, and 2-benzohellicin gave a canary yellow spot.



2-Benzohellicin

FURTHER ROTATION STUDIES ON POPULIN AND TREMULOIDIN

Because the original rotation results on tremuloidin noted earlier in this report indicated some mutarotation in pyridine, the use of another solvent was investigated. In a solubility study of tremuloidin in acetone-water systems it was found that 80% acetone was a good solvent for tremuloidin and gave a clear solution for rotation studies, even at relatively high concentrations. A sample of pure tremuloidin (fraction D-2 of Figure 4, melting at 206-207°) was dissolved in 80% acetone and had a specific rotation $[\alpha]_D^{25} -12.3$ ($c = 1.5$ in 80% acetone). Standing for 72 hours did not affect the rotation. The same product was dissolved in pyridine and gave a specific rotation $[\alpha]_D^{25} +17.1^\circ$ ($c = 3.1$ in pyridine). After standing 72 hours, the pyridine solution turned somewhat yellow and gave a specific rotation $[\alpha]_D^{25} +19.5^\circ$. The pyridine was evaporated from the yellow solution, and the residue was dissolved in 80% acetone.

This solution gave a specific rotation for tremuloidin identical with that of the original tremuloidin in 80% acetone. Thus, the same material that rotates -12.3° in 80% acetone rotates $+17.1^\circ$ in pyridine.

The rotation of synthetic populin was also determined in 80% acetone and found to be $[\alpha]_D^{25} -29.7^\circ$ ($c = 5$ in 80% acetone), and this value did not change with time. This rotation compares with $[\alpha]_D^{25} -2.4^\circ$ ($c = 4.5$ in pyridine) for the same product. The rotation in pyridine did not change with time either.

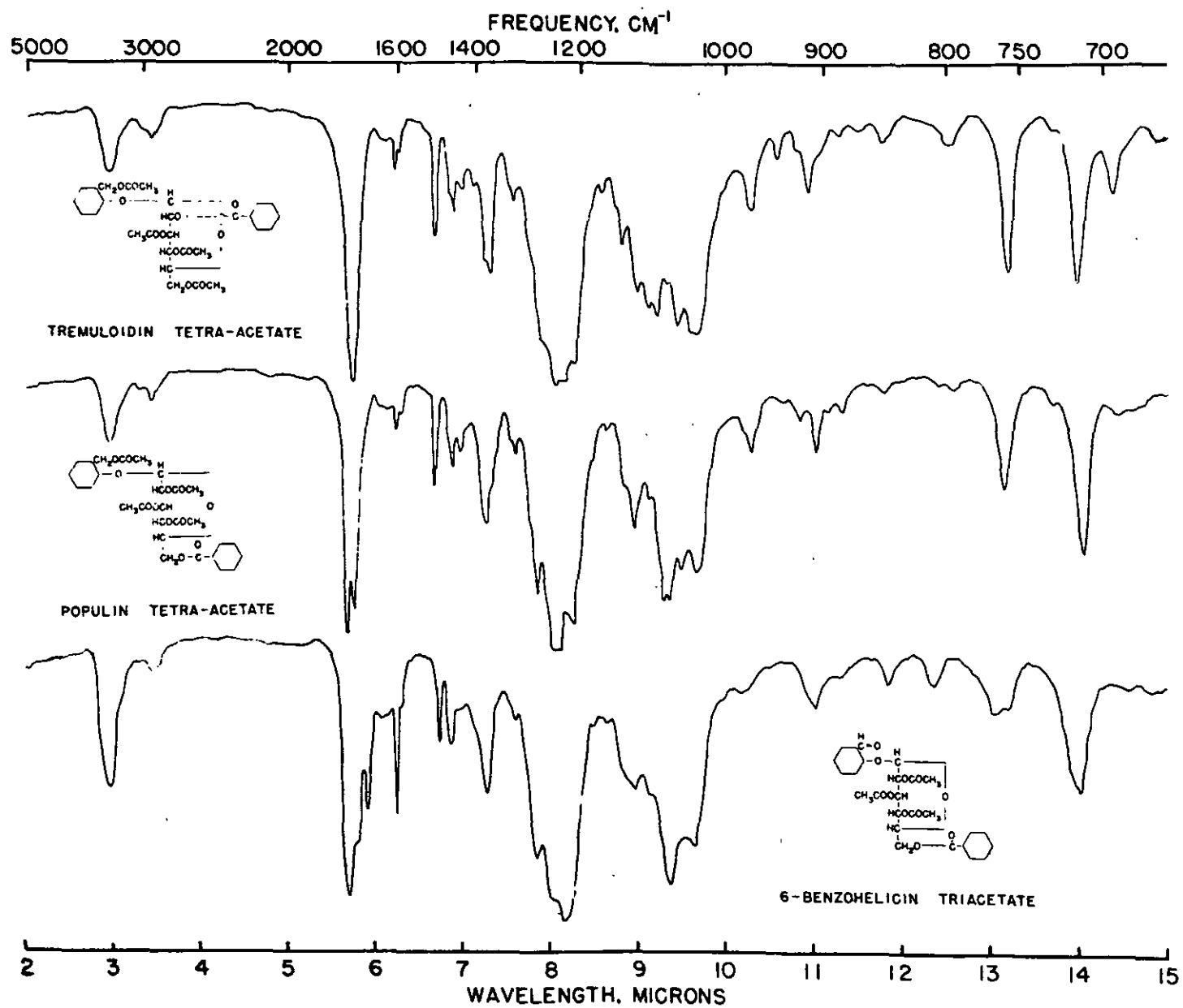
INFRARED SPECTRA

Infrared spectra of all known compounds were determined. In addition infrared spectra of unknown glucosides and sugars were determined and compared. The spectra are attached to this report.

LITERATURE CITED

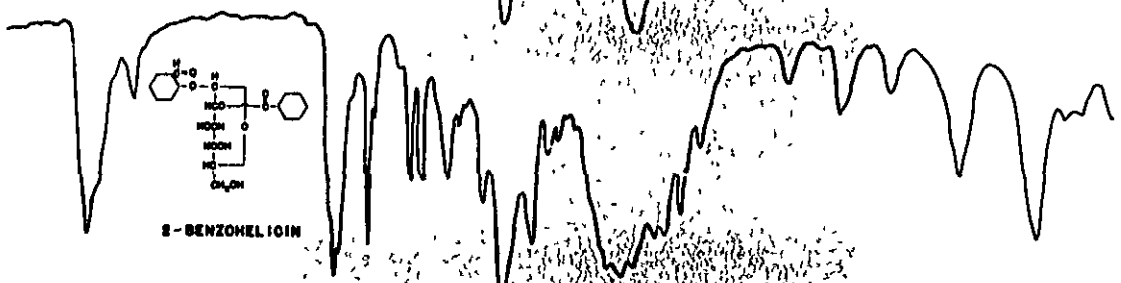
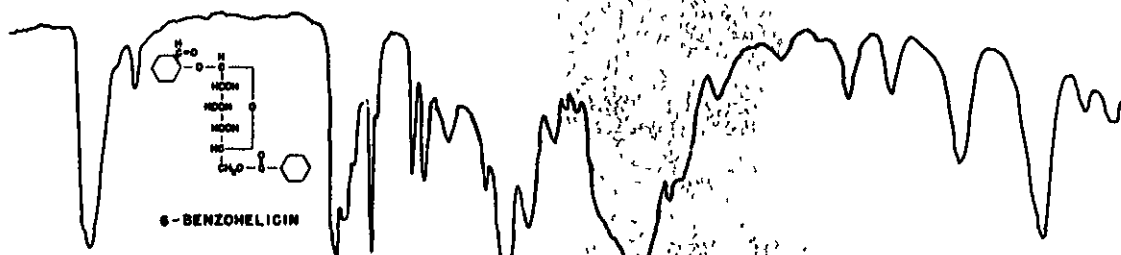
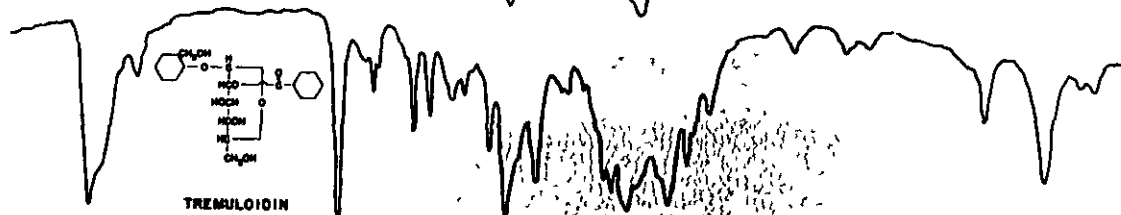
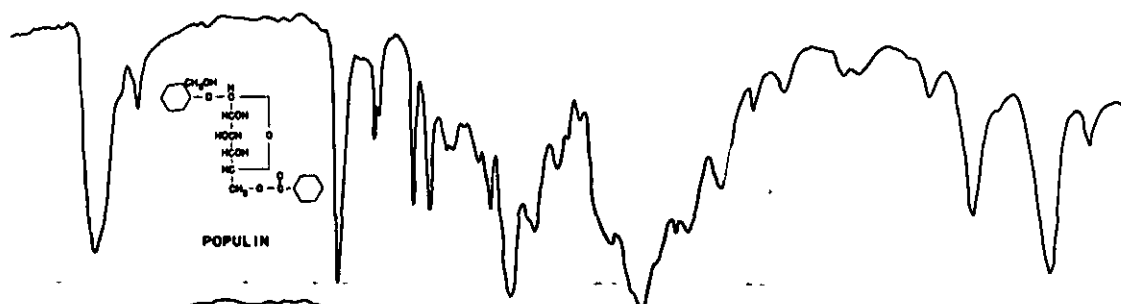
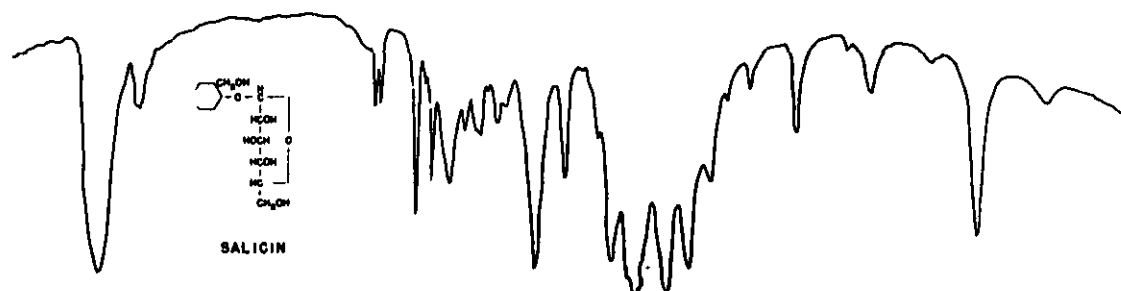
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FREQUENCY, CM^{-1}

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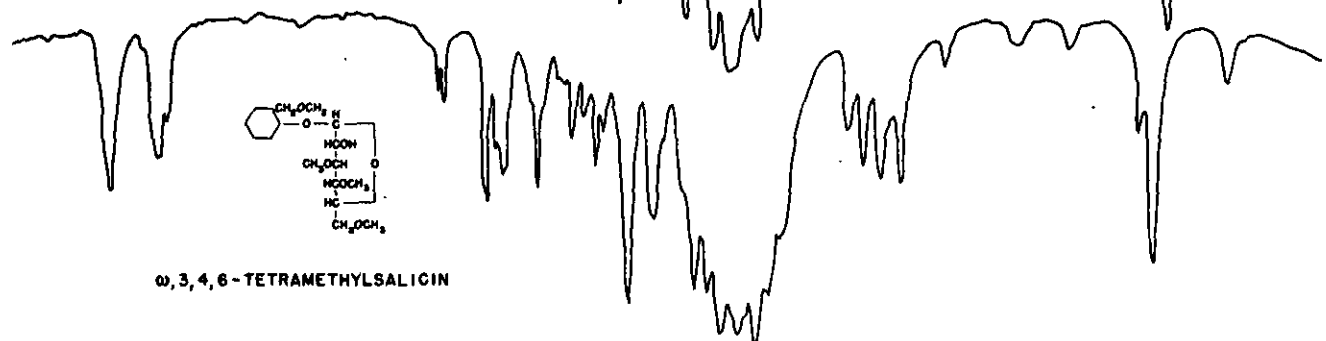
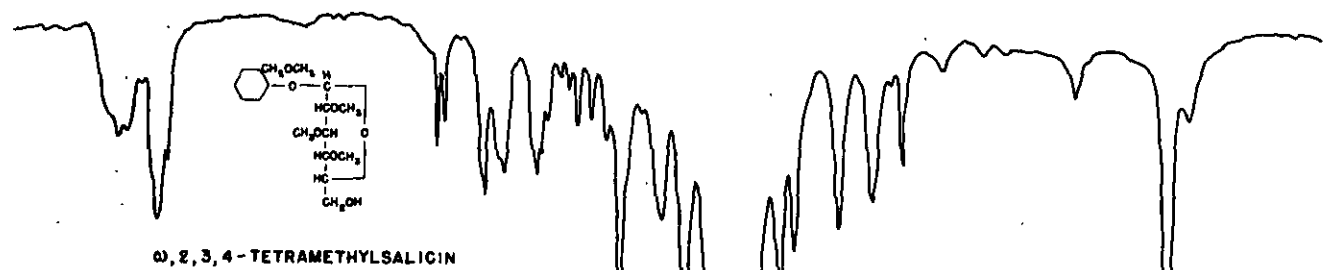
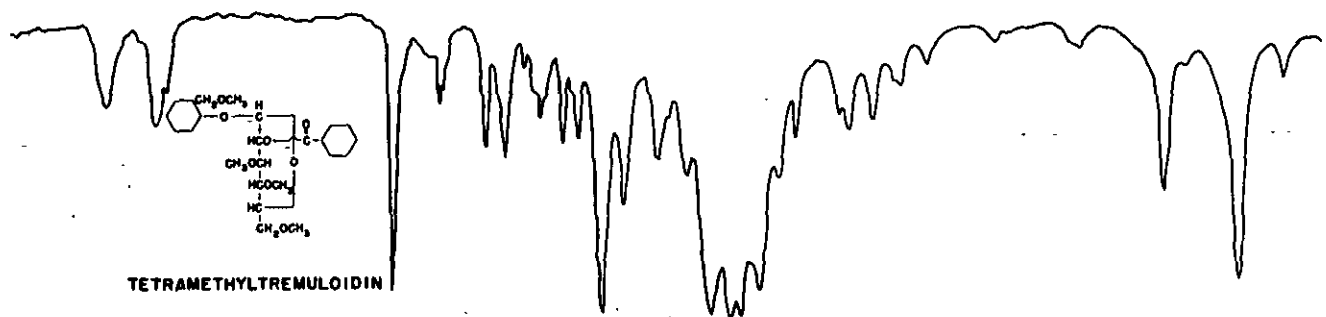
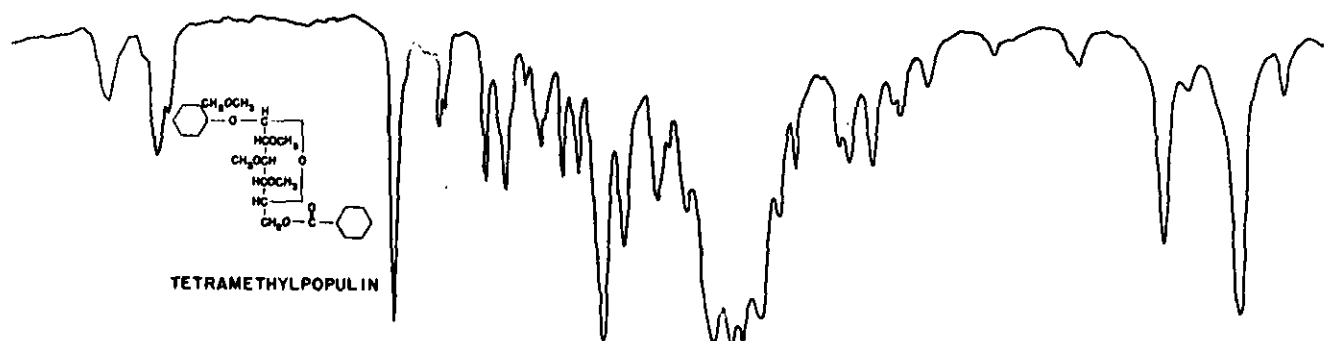


WAVELENGTH, MICRONS

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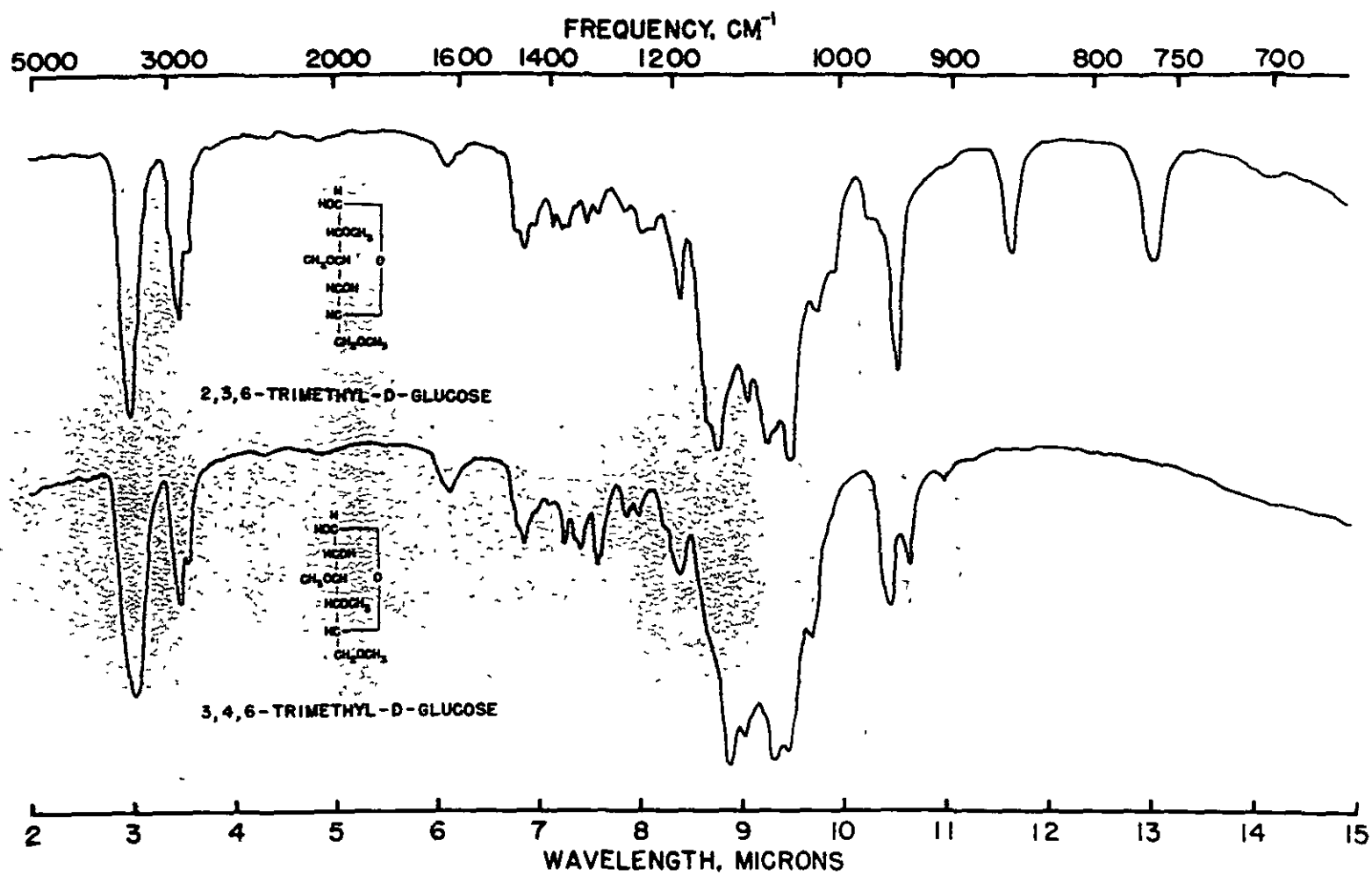
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PROJECT REPORT FORM

PROJECT NO. 1932
COOPERATOR IPC
REPORT NO. 2
DATE September 6, 1956
NOTE BOOK 1359
PAGE 114 TO 122
SIGNED Donald L. Beyer
Donald L. Beyer

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Dr. Pearl (2)
Mr. Beyer

CAUSTIC HYDROLYSIS OF SPRUCE AND OF ASPEN HEMICELLULOSE

Since the discovery of p-hydroxybenzoic acid in Populus tremuloides, we are endeavoring to find out to what extent the acid is present. In Project 1932 Report No. 1, a number of different types of aspen sawdust were treated in various manners and each contained the acid. The caustic hydrolysis appeared to produce the largest amounts of the acid, so for individual test experiments this procedure is followed.

Hemicellulose

A sample of hemicellulose was obtained from Mr. David Lea carrying his code number W-D5. The sample was prepared November 7, 1952. The sample was aspenwood hemicellulose extracted by direct extraction with 5% potassium hydroxide.

Five grams of the above mentioned hemicellulose was boiled in 150 cc. of N sodium hydroxide for 8 hours. When cool, the caustic solution was extracted with ether. This ether solution was evaporated to dryness, leaving only a little waxy solid which showed nothing to any of our phenol sprays.

The caustic solution was acidified with dilute sulfuric acid and was again extracted with ether. This ether solution was concentrated to a small volume and bottled. Chromatographing of the sample showed nothing but a faint R_f 0.00 in all developers.

The remaining aqueous solution was also chromatographed, showing two materials:

BuOH 2% aqueous ammonia, R_f - 0.00; 0.02

BuOH, Pyridine, H_2O (10:3:3) R_f - 0.00, 0.11

Apparently no p-hydroxybenzoic acid is present.

It was decided to check a soft wood to see if any p-hydroxybenzoic acid was present. A sample of spruce sawdust was obtained from the pulp lab. to be used for this experiment. The sawdust contained 6.51% moisture.

A sample of 107 grams (100 grams o.d.) of spruce sawdust was boiled under reflux for 8 hours in 3000 cc. of 4% sodium hydroxide. The mixture was stirred during the boiling period by means of a magnetic stirrer. When cool, the mixture was filtered and washed with water. The sawdust was washed with acetic acid, ethanol, and ether respectively, air dried and stored. The sawdust amounted to 75 grams o.d. basis. The washings were discarded.

The filtrate was acidified with dilute sulfuric acid and extracted with ether. The ether solution was concentrated to a small volume and chromatographed.

Results

BuOH 2% aqueous ammonia.

R_f - 0.00; 0.10; 0.37; 0.73; 0.86; 0.91

10:3:3 Butanol-pyridine-water

R_f - 0.17; 0.28; 0.36; 0.44; 0.56; 0.79

Vanillic acid - R_f 0.44. p-Hydroxybenzoic acid - R_f 0.56

BuOH saturated with water, using a paper soaked in a buffer solution pH 6.6.

R_f - 0.04; 0.12; 0.18; 0.22; 0.44; 0.53; 0.58; 0.67; 0.80;
0.84; 0.92

Vanillic acid R_f - 0.53. p-Hydroxybenzoic acid R_f - 0.67

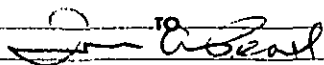
Vanillic acid was positively identified by comparison both by position and color. The p-hydroxybenzoic acid however, could not be positively identified. A very small spot occurred to correspond with the authentic sample, but the color did not seem to match. The spot however, was much too small to accurately identify. If p-hydroxybenzoic acid is present it is there in extremely small amounts.

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Notebook 1739:
12-14, 16-17

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COOPERATOR I.P.C.
REPORT NO. 2
DATE January 9, 1959
NOTE BOOK 1633 and 1739
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SIGNED  Irwin A. Pearl

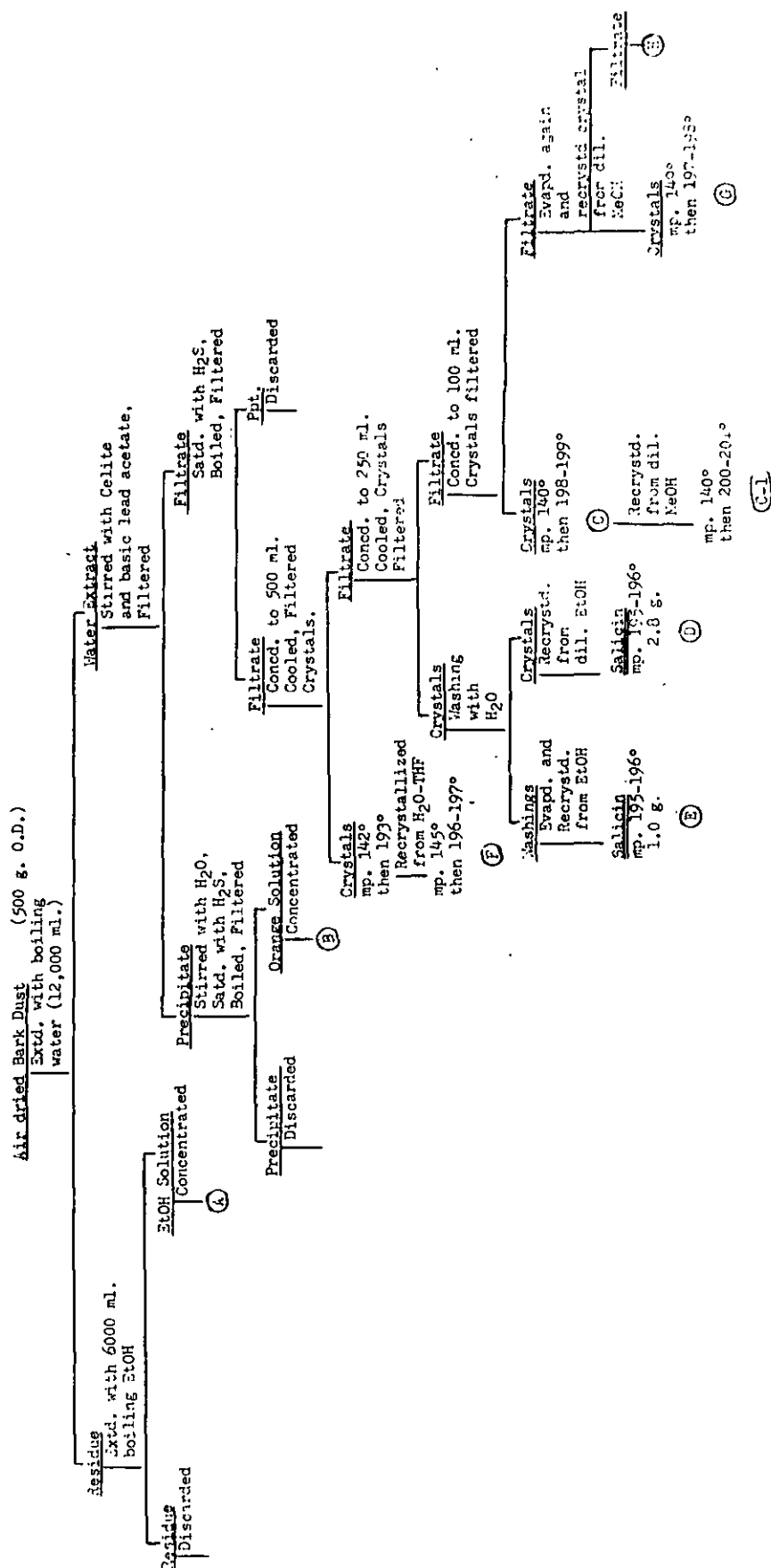
FURTHER STUDIES ON THE GLUCOSIDES OF VARIOUS POPULUS SPECIES

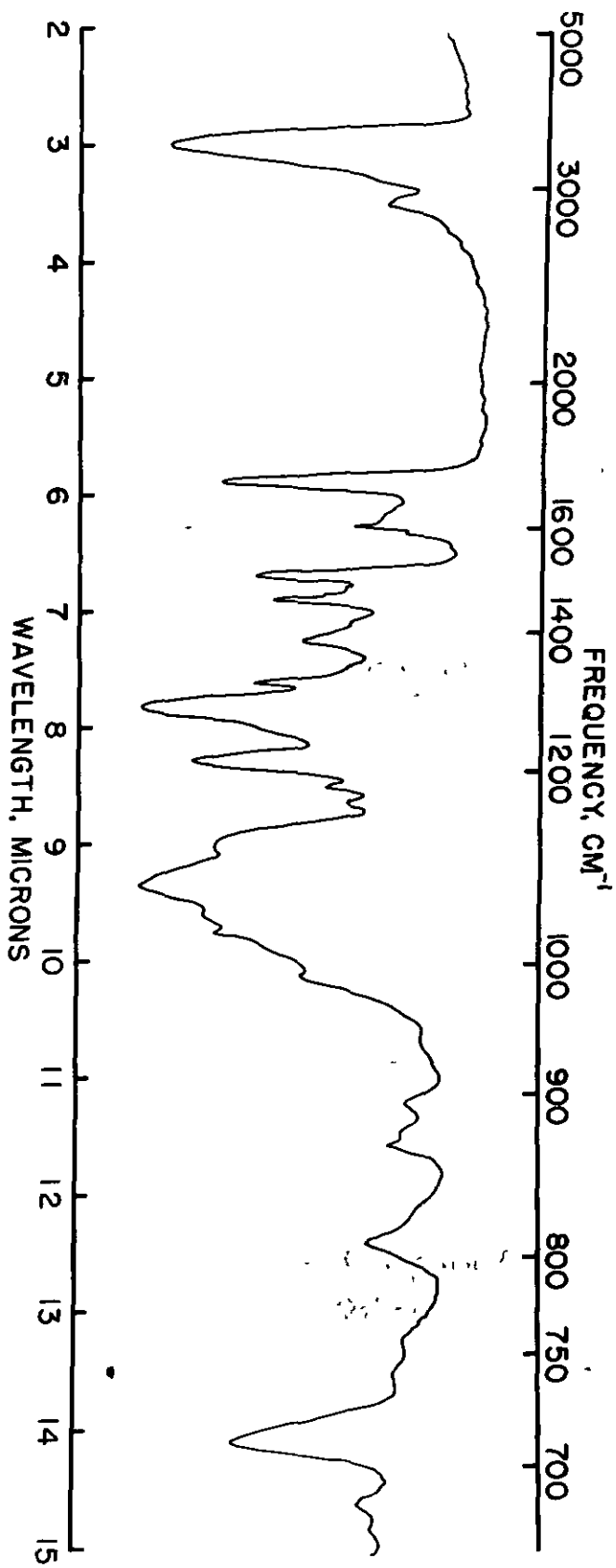
In Report No. 1 a number of extractions of the bark of Populus tremuloides were reported including flow sheets describing the fractionation schemes and identifying the fractions isolated. Salicin, populin, and tremuloidin were identified in P. tremuloides bark extracts and several unidentified glucosides were indicated. The present report describes further work on some of the isolated fractions and on the extracts of some other Populus species.

AQUEOUS EXTRACTION OF POPULUS ALBA BARK--On page 18 of Report No. 1 the isolation of authentic populin from Populus alba leaves was described. The bark of the same Populus alba tree was dried, extracted with hot water, and processed in accordance with the flow sheet of Figure 1. From this flow sheet it is apparent that except for salicin, the bulk of the crystalline material appears to be a product which melts a little below 150° and then solidifies and remelts at approximately 200°. Fraction G was employed for rotation and found to give $[\alpha]_D^{25} - 37.3^\circ$ (c = 1 in pyridine). The solution employed for rotation was evaporated to dryness, and the residue was recrystallized from water to give colorless needles melting at 149°, resolidifying and then melting at 200-201°. This sample was submitted for infrared absorption spectrum, and the curve is shown in Figure 2.

Figure 1

Fractionation of Populus alba Bark





Infrared absorption spectrum of new glucoside suspected of being Salireposide.

The infrared absorption spectrum was entirely different from that of either populin or tremuloidin. Accordingly, this material could not be a mixture of the two known glucosides.

Fraction E, F, and G, and H were chromatographed along with pure salicin, populin, and tremuloidin and developed with 9:2:2 ethyl acetate-acetic acid-water. The papers were sprayed with the modified silver spray described in Report No. 1. Fraction E proved to be salicin, but fractions F and G did not match with any of the knowns spotted. Similar results were obtained with the 10:3:3 butanol-pyridine-water developer. The new unknown glucoside had a lower R_f than populin in both solvent systems. Fraction H contained spots for four sugars, salicin, populin, but no tremuloidin or the new glucoside.

Salicin, populin, tremuloidin, and Fractions C-1, F, and G were each treated on a spot plate with sulfuric acid and nitric acid for their color reactions. The test was made by placing one milligram of the material in the spot plate depression and treating with six micro drops of concentrated sulfuric acid. The color was noted. Then one micro drop of nitric acid (sp. g. 1.30) was added, and the color again noted. The results are shown in Table I.

TABLE I
COLOR REACTIONS OF GLUCOSIDES

<u>Glucoside</u>	<u>Sulfuric Acid</u>	<u>Nitric Acid</u>
Salicin	Bright red	Brown precipitate
Populin	Brighter red	Brown precipitate
Tremuloidin	Bright red	Pale brown precipitate
Fraction C-1	Pale brown	Clear orange-yellow fluorescent solution
Fraction F	same as C-1	same as C-1
Fraction G	same as C-1	same as C-1

Thus, all data obtained on the unknown glucoside material melting first at about 150° and then again at about 200° indicate that it must be a new glucoside. Melting characteristics and specific rotation suggest that it may be identical with salireposide isolated from Salix repens by Wattiez (1) and from Salix purpurea L. subspecies angstifolia Koidz by Sakai and co-workers (2). As noted in Report No. 1 on page 13 Fujikawa and Akimasa (3) demonstrated that salireposide was a benzoylated glucoside of 2,5-dihydroxybenzyl alcohol (gentisyl alcohol). These authors also showed that debenzoylated salireposide was hydroxysalicin and assumed that salireposide must be hydroxypopulin. However, the location of the benzoyl substitution on the glucose was not demonstrated and other structures for salireposide are possible. These structures include monobenzoates of 2-gentisyl alcohol- β -D-glucoside with benzoyl substitution on any of the possible glucose carbon atoms.

Further reference to the unknown glucoside isolated from Populus alba bark will be made in other studies described in this report.

AQUEOUS EXTRACTION OF POPULUS TREMULA BARK--Populus tremula bark obtained from Dr. Dean Einspahr on May 26, 1958 was extracted with water in the same manner as that described for Populus alba. The aqueous extract was processed in the same manner to the concentration of the purified extract stage. The entire concentrated solution was chromatographed along with the fractions of Populus alba noted above. The spots noted corresponded with those of Fraction H of Populus alba which contained spots for four sugars, salicin, populin, but no tremuloidin or the new glucoside tentatively identified as salireposide.

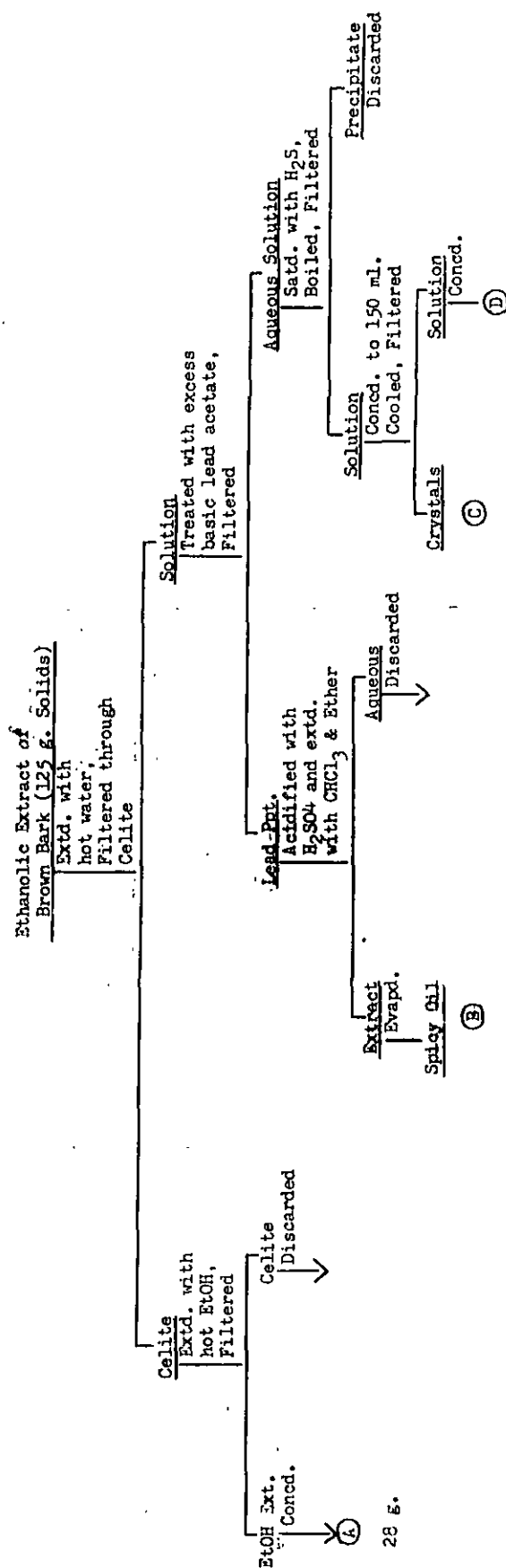
POPULUS GRANDIDENTATA BARK--A large batch of bigtooth aspen (Populus grandidentata) bark was obtained on July 9, 1958 from Dr. Nicholas Jappe. Data for the tree was supplied by Miss Barbara Reeder as follows: The tree was cut on July 8, 1958 from Clone No. 34, Ripco Farm, Eagle River, Wisconsin. The tree was 34 years of age, 60 ft. in height, and had a base diameter of 20.2 cm. The total bark comprised brown broken up material near the base of the tree and greenish smooth material higher up on the tree. The two types of bark were kept separate.

The green smooth bark was allowed to air dry and was then broken into small pieces, milled in the Wiley mill, air dried, and bottled.

The brown material was broken into small pieces and extracted directly with ethanol at room temperature. The ethanol extract was concentrated to a small volume. An amount containing 125 grams of solids was evaporated to dryness in a rotating evaporator. The residue was boiled with 2.5 liters of water and allowed to stand overnight. The mixture was filtered through Celite and processed in accordance with the flow sheet of Figure 3. The first crystalline product from the purified aqueous extract (Fraction C) was chromatographed in the 10:3:3 butanol-pyridine-water developer, and the chromatogram was sprayed with the modified silver spray. Spots for salicin, tremuloidin and four sugars were noted, but no populin was found.

POPULUS TACAMAHACA BARK--A large batch of balsam poplar (Populus tacamahaca) bark was obtained from Miss Barbara Reeder on August 4, 1958.

Figure 3
Preliminary Fractionation of Ethanol Extractives of Populus grandidentata



Data supplied for the tree was as follows: The tree was cut on August 1, 1958 on the Ontanogon River, base of Military Hill on west side of road. The tree was 30 years of age, 57 feet in height, and had a base diameter of 17.2 x 19.2 cm. The bark as allowed to air dry and was Wiley milled on August 12, 1958. The milled bark was air dried and bagged in polyethylene on August 15, 1958 with a moisture content of 7.9%.

About 100 grams of the fresh bark was broken into small pieces and boiled with 600 ml. of water. The mixture was filtered, and the clear tea-colored filtrate was treated with excess basic lead acetate. The precipitate was filtered with the aid of Celite, and the clear filtrate was saturated with hydrogen sulfide. The mixture was boiled and filtered. The colorless filtrate was concentrated to a syrupy liquid in a rotating evaporator. The syrup was diluted somewhat and spotted on Whatman No. 1 paper along with authentic salicin, populin and tremuloidin. The chromatogram was developed overnight with 10:3:3 butanol-pyridine-water, and the dried chromatogram was sprayed with the modified silver spray. The sprayed chromatogram indicated five sugars and four glucosides in the P. tacamahaca extract. One of the glucosides was salicin. One spot was located below salicin, but not as far as populin, one spot opposite populin, and one spot much lower than tremuloidin. This last spot may not be a glucoside because it gave a dark spot when sprayed with silver nitrate before treatment with alkali.

Because the modified silver spray did not differentiate individual glucosides from phenolic materials or from sugars the purified P. tacamahaca extract along with the three authentic glucosides was chromatographed in

replicate in both the 9:2:2 ethyl acetate-acetic acid water and in the 10:3:3 butanol-pyridine-water developers. The developed papers were sprayed with para-anisidine, diazotized para-nitroaniline, and modified silver spray reagents.

The ethyl acetate-acetic acid-water chromatogram showed only four sugars when sprayed with para-anisidine. When sprayed with diazotized para-nitroaniline, no sugars were indicated; a faint red spot appeared for salicin and a similar spot appeared for the extract; no spot appeared for populin, but a faint blue spot appeared at the same R_f for the extract; and no spot for tremuloidin, but a very strong red spot for the P. tacamahaca extract at the same R_f . These red spots had blue centers. The modified silver spray showed spots for the extract even before spraying with alkali at the same R_f as tremuloidin which appeared identical with the red spot with blue center obtained with the diazotized para-nitroaniline spray. After spraying with alkali, the silver sprayed chromatograms showed spots for four sugars and all the other spots noted with the other spray reagents. Some streaking of the extract took place in this developing system.

The butanol-pyridine-water system gave better development and resolution of spots. The silver spray indicated for the bark extract sugars at R_f 's 0.01, 0.02, 0.06, 0.12, 0.17, and 0.22; a spot at 0.38; and four spots in the glucoside region with R_f 's 0.51, 0.59, 0.70, and 0.79. The diazotized para-nitroaniline spray which indicates phenolic materials and not glucosides or sugars indicated a reddish spot at R_f 0.59, a lavender spot at 0.70 and a red spot with blue center at 0.79. The R_f values for the known glucosides in this solvent system at 25° were: salicin, 0.52;

populin, 0.69; and tremuloidin, 0.77. Thus, the bark extract appears to contain only one glucoside--salicin--and three phenolic substances. It is also possible that the phenolic materials might overlay glucosidic material, and it is further possible that the phenolic spots may be caused by glucosides with free phenolic groups. Salireposide would be such a glucoside.

The purified glucoside isolated from Populus alba melting at 150° and then again at 200° and suspected of being salireposide was spotted along with authentic salicin, populin, and tremuloidin and developed in the 10:3:3 butanol-pyridine-water developer. The modified silver spray of the chromatogram indicated all authentic glucosides with the same R_f values noted above and the new glucoside with an R_f of 0.68, almost identical with that of populin. The diazotized para-nitroaniline spray indicated only the new glucoside as a lavender spot at R_f 0.68. Thus it appears that the new glucoside contains a free phenolic group. Since its R_f value in the solvent systems employed is essentially the same as that of populin, some other developers or methods would be necessary to separate the two.

REINVESTIGATION OF BARK EXTRACTS--Fresh hot water extracts were made from the dried powdered barks of Populus alba, P. tremula, P. tacamahaca, and P. tremuloides. These were filtered through Celite and concentrated, but were not purified by means of basic lead acetate. The crude concentrated aqueous extracts were spotted along with known glucosides and developed in the 10:3:3 butanol-pyridine water developer. Papers were sprayed with both the silver and diazotized para-nitroaniline sprays.

The chromatograms for all the barks were very similar. Silver spraying indicated all the sugars noted earlier for P. tacamahaca, but only in the case of the tacamahaca was the R_f 0.22 spot real heavy. The spot at 0.38 gave a lavender color with the diazo spray. The spot at the salicin R_f of 0.52 gave a very strong silver spot and a very weak red diazo spot indicating salicin in the barks with a trace of some phenolic material at the same point. A weak silver spot and a red diazo spot appeared at R_f 0.59, and a strong lavender spot appeared at R_f 0.69 identical with and at the same point as the new glucoside suspected of being salireposide. Populin also gave a spot with silver at this same R_f . Weak silver spots appeared at the R_f of tremuloidin (0.77), but no spots appeared at this R_f with the diazo spray. Heavy silver spots appeared (even before spraying with alkali) at R_f 0.79, and these spots also appeared on the diazo sprayed sheets as intense red spots.

Thus, it appears that the ~~crude~~ extracts of these four Populus species contain essentially the same glucosides. However, it should be noted that the relative amounts in the various extracts are different, and this difference in relative amounts causes minor differences in R_f values for the similar spots on the chromatograms. Reported R_f values are average values.

The finding of suspected salireposide in fresh crude aqueous extract of Populus tremuloides led to a reinvestigation of some of the fractions isolated in earlier studies and reported in Report No. 1. On page 32 of the earlier report the continuous ether extraction of the

concentrated aqueous extract of Populus tremuloides after removal of populin and tremuloidin was described. During this extraction a number of fractions of crystalline material melting at approximately 150° and then again at 200° were obtained. Fractions L-1 and N-1 from Figure 5 on page 32 of Report No. 1 were chromatographed along with the new glucoside (suspected salireposide) from Populus alba in the butanol-pyridine-water developer and sprayed with diazotized para-nitroaniline. The lavender spot at R_f 0.69 was identical for all materials. Further work on the new glucoside obtained from these fractions will be reported later.

SALICYL ALCOHOL STUDIES--During the investigations noted above it became evident that salicin solutions which gave only one spot when fresh gave a sugar spot and a phenolic spot when allowed to stand for some time. Accordingly, authentic salicyl alcohol was spotted and developed in the butanol-pyridine-water and the ethyl acetate-acetic acid-water systems. Silver spraying gave a good spot and diazotized para-nitroaniline spraying gave a bright red spot only after alkali treatment with butanol-pyridine-water R_f 0.79 and ethyl acetate-acetic acid-water R_f 0.95.

Salicyl alcohol was spotted along with purified aqueous extracts from Populus tremuloides, alba, and tacamahaca and the chromatograms developed in both previously used solvents and sprayed with both the modified silver and the diazotized para-nitroaniline sprays. The silver spray indicated spots for the salicyl alcohol and all the extracts at the R_f of salicyl alcohol, but only for the extracts before treatment with alkali. The diazo spray gave red spots for all materials. In the case of the P. alba and P. tacamahaca spots, the red spots had blue centers.

Thus it appears that salicyl alcohol may be responsible for the red spot at butanol-pyridine-water R_f 0.79 and ethyl acetate-acetic acid-water R_f 0.95, but some other material with the same R_f values in these two developing systems must be responsible for the immediate silver spot before treatment with alkali and/or the blue center in the red diazo spot. Inasmuch as all bark extracts appeared to contain the material giving the immediate silver spot, it was desired to determine whether salicyl alcohol was probably present in all of these extracts.

A fresh and an aged salicin solution were spotted on paper along with authentic salicyl alcohol and authentic glucose. The chromatograms were developed in 10:3:3 butanol-pyridine-water and sprayed with both the modified silver and the diazotized para-nitroaniline sprays. Fresh salicin solution gave only one spot with silver and no spot with the diazo spray. Aged salicin solution gave a spot for salicin and spots for glucose and for salicyl alcohol with the silver spray and only a spot for salicyl alcohol with the diazo spray. Therefore, it is apparent that any bark extract containing substantial amounts of salicin will certainly contain some salicyl alcohol due to hydrolysis of the salicin.

Because it was found that salicyl alcohol did not reduce silver nitrate before addition of alkali a dihydric phenol was tried. Hydroquinone was spotted on paper, and the paper was dried and bathed in an acetone solution of silver nitrate. The spot appeared as a gray silver spot very similar to the spots obtained from the Populus barks at butanol-pyridine-water R_f 0.79. Thus, it appears that some dihydric phenolic material or

similar reducing substance was responsible for these spots. 2,5-Dihydroxybenzyl alcohol, the aglucone of salireposide, would be such a compound, and would in all probability be present in these bark extracts if the lavender spot at butanol-pyridine-water R_f 0.69 did prove to be salireposide. Synthetic studies are underway on the preparation of 2,5-dihydroxybenzyl alcohol, and chromatographic studies will be reported in the future.

PRELIMINARY STUDIES ON SUGARS IN POPULUS BARKS--Purified aqueous extracts of barks of Populus alba, P. tremula, P. tremuloides, and P. tacamahaca were spotted along with known glucose, fructose, and sucrose and developed in 9:2:2 ethyl acetate-acetic acid-water. The papers were sprayed with both urea and para-anisidine spray reagents. The para-anisidine spray indicated only glucose and sucrose (R_g 0.61) in all of the extracts. The urea spray indicated fructose (R_g 1.34), sucrose (R_g 0.61) and two oligosacharrides with R_g 's 0.07 and 0.20 in all of the extracts.

Unpurified hot water extracts of Populus tremuloides and P. tacamahaca barks were chromatographed along with known glucose, fructose, and sucrose in the ethyl acetate-acetic acid-water developer. The chromatograms were sprayed with modified silver, diazotized para-nitroaniline, urea, and para-anisidine sprays. The diazotized para-nitroaniline spray indicated nothing in the sugar area. The silver spray indicated glucose, fructose (R_g 1.33), sucrose (R_g 0.60), and spots at R_g 's 0.43, 0.29, 0.17, and 0.07 for both bark extracts although the P. tacamahaca extract appeared to have more of all sugars. In addition, the P. tacamahaca extract contained materials at R_g 0.75 and R_g 1.63 not present in the P. tremuloides. The urea spray for ketoses indicated only fructose,

sucrose and the two oligosacharrides at R_g 0.07 and 0.17. Para-anisidine indicated glucose, sucrose, the spot at R_g 1.63 and at R_g 0.29, the former only in the P. tacamahaca extract.

FRACTIONATION OF FRESH POPULUS TREMULOIDES BARK EXTRACT ON ACTIVE CARBON--One hundred grams of ground dried Populus tremuloides bark was boiled with 500 ml. of water and filtered. The filtrate was purified by means of basic lead acetate and hydrogen sulfide as described in the past. The colorless filtrate was concentrated in the rotating evaporator to pale tan syrup. This syrup was dissolved in 250 ml. of water, treated with 22.5 grams of Darco G-60 active carbon, and stirred for one hour. The stirred mixture was treated with 20 grams of Fibriflo filter aid, stirred thoroughly, and filtered on a sintered glass funnel. The residue on the funnel was sucked dry and then washed successively with 500 ml. volumes of solvents as noted in Table II. Each of the six fractions noted in Table II was concentrated to a few milliliters in the rotating evaporator, and the concentrated solutions were spotted along with the original extract. Spots were located by means of the modified silver spray. Results are noted in Table II.

TABLE II

ACTIVE CARBON FRACTIONATION OF P. TREMULOIDES BARK EXTRACT

<u>Fraction</u>	<u>Solvent</u>	<u>Chromatographic results</u>
1	Water	High concentration of sugars and one compound between sugars and glucosides. No glucosides.
2	1% EtOH	Less sugars; still no glucosides.
3	5% EtOH	Still less sugars; no glucosides.
4	25% EtOH	A trace of sugars; considerable salicin; no other glucosides.
5	50% EtOH	A trace of sugars; considerable salicin; no other glucosides.
6	50% Pyridine	No sugars; a little salicin; two glucosides in quantity.

The results of Table II indicate that the components of Populus bark extracts can be separated by means of absorption on active carbon followed by selective elution. More studies on these separations will be reported in the future.

LITERATURE CITED

- (1) M. N. Wattiez, Bull. Soc. Chim. Biol. 13, 658 (1931).
- (2) S. Sakai, M. Tsurumi, Y. Eno, and F. Inukai, Bull. Inst. Phys. Chem. Research (Tokyo) 22, 868 (1943).
- (3) F. Fujikawa and A. Tokuoka, J. Pharm. Soc. Japan 67, 121 (1947).

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PROJECT REPORT FORM

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REPORT NO. 3
DATE September 10, 1956
NOTE BOOK 1359
PAGE 134 TO 141
SIGNED Donald L. Beyer
Donald L. Beyer

Copies to: Files
Dr. Pearl (2)
Mr. Beyer

ACID HYDROLYSIS OF ASPEN SAWDUST

As a further study on the presence of p-hydroxybenzoic acid in Aspen, two experiments were performed hydrolyzing Aspen sawdust and Aspen sawdust previously hydrolyzed with caustic with an acid.

Both hydrolyses were run in the same manner. A sample of 142 grams (100 grams o.d.) of Aspen sawdust was stirred and boiled under reflux for 8 hours in a solution of 150 grams of sulfuric acid in 3000 cc. of water. For the second experiment, 60 grams of o.d. Aspen sawdust recovered from a caustic hydrolysis was boiled in 90 grams of acid in 1800 cc. of water. The Aspen sawdust carried our number 1932-38-A.

The sawdust recovered from a caustic hydrolysis carried our number 1359-95-C.

When cool, the mixture was filtered. The filtrates were extracted with ether. The ether solution was concentrated to a small volume and bottled. The sawdust was washed well with water, then with ethanol and finally with ether. After air drying, the sawdust was bottled. The washings were discarded.

"A" will denote the extract from the Aspen sawdust.

"B" will denote the extract from the recovered sawdust from a caustic hydrolysis.

Yields:

A. 0.71 grams, 0.71%

B. 0.36 grams, 0.6%

Both extracts were chromatographed with the following results:

BuOH-2% aqueous ammonia.

A. Benzidine R_f - 0.00; 0.08; 0.10; 0.35; 0.42; 0.75; 0.81

2,4 R_f - 0.35; 0.42

$FeCl_3$ R_f - 0.00; 0.08; 0.10

Maule R_f - 0.08; 0.35; 0.81

B. Benzidine R_f - 0.01; 0.08; 0.10; 0.35; 0.42; 0.75

R_f 0.35 is syringaldehyde and R_f 0.42 is vanillin.

BuOH, pyridine, water (10:3:3).

A. Benzidine R_f - 0.34; 0.44; 0.56; 0.80; 0.83

2,4 R_f - 0.80; 0.83

$FeCl_3$ R_f - 0.56

Maule R_f - 0.34; 0.80

B. Benzidine R_f - 0.34; 0.44; 0.56; 0.66; 0.80; 0.83

R_f 0.34 is syringic acid

R_f 0.44 is vanillic acid

R_f 0.56 is p-hydroxybenzoic acid

The two samples were spotted and chromatographed for determining the quantitative yields of the five known materials. For vanillin and syringaldehyde, the papers were developed with n-butyl ether saturated with water. For the three acids, the papers were developed with benzene saturated with formic acid. The known materials were cut from the paper, eluted with ethanol, treated with alcoholic potassium hydroxide and the density reading was obtained from the spectrophotometer.

The wavelength used for each sample as well as the conversion factor for converting the density into mg./liter is as follows:

Vanillin wavelength is 352 conversion fraction is 5.33

Syringaldehyde wavelength is 368 conversion fraction is 6.76

Vanillic acid wavelength is 298 conversion fraction is 13.1

Syringic acid wavelength is 300 conversion fraction is 16.4

p-Hydroxybenzoic acid wavelength is 278 conversion fraction 8.30

Results were as follows:

Vanillin:	A - contained 10.9 mg., 1.5%
	B - contained 15.5 mg., 4.3%
Syringaldehyde:	A - contained 14.4 mg., 2.0%
	B - contained 11.4 mg., 3.2%
Vanillic acid:	A - contained 26.6 mg., 3.7%
	B - contained 10.0 mg., 2.8%
Syringic acid:	A - contained 36.8 mg., 5.2%
	B - contained 13.8 mg., 3.8%
p-Hydroxybenzoic acid:	A - contained 127.1 mg., 18.0%
	B - contained 13.8 mg., 3.8%

PROJECT REPORT FORM

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DATE March 31, 1961
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Pearl (2) pages 100-118
Reading Copy 120-127
130-140
152-156

Notebook 1834
pages 29 and 53

Irwin A. Pearl

SALIREPOSIDE FROM THE BARK OF POPULUS TREMULOIDES

In Project Report No. 1, the isolation of populin from the bark of the American quaking aspen (Populus tremuloides), and in Project Report No. 2, the isolation of a glucoside suspected of being salireposide was reported. The present report is concerned with the identification of the new glucoside as salireposide. Experiments reported were performed by both Dr. S. F. Darling and myself.

Having found in Report No. 2 that a glucoside suspected of being salireposide present in the bark of European white poplar (Populus alba) gave a purple spot with diazotized p-nitroaniline, a number of unidentified glucoside fractions isolated from the bark of Populus tremuloides were subjected to paper chromatography and spraying with the diazotized p-nitroaniline spray. These fractions were reported in Report No. 1. Two of the fractions first studied were Fraction D-1 of Figure 2 in Report No. 1 and the product described on page 29 of Report No. 1 as crystals melting at 153-156°, resolidifying, and melting at 199°. These were spotted on paper along with synthetic populin, the "populin" fraction originally isolated from Populus tremuloides (reported in Project Report 1, page 37), and the suspected salireposide from Populus alba. These papers were developed in 10:3:3 butanol-pyridine-water (BPW) and in 9:2:2 ethyl acetate-acetic acid-water (EAW), and the developed papers were sprayed

with both the diazotized p-nitroaniline (DpNA) and modified silver (Ag) spray reagents described in Reports No. 1 and 2. Of all the compounds spotted, only synthetic populin did not give a purple spot with DpNA at BPW R_f 0.69 and EAW R_f 0.84. All compounds gave a black spot with Ag at these same R_f values. Thus it appears that suspected salireposide and populin have the same R_f values in these two solvent systems, but only the suspected salireposide gives the purple color with DpNA. Furthermore, it appears that the "populin" fraction isolated from Populus tremuloides was not populin at all, but was actually the suspected salireposide. The reported mixed melting point determination with populin reported on page 37 of Project Report 1 was repeated several times, and in the repeat experiments, a noticeable depression was observed when the unknown crystals were mixed with authentic synthetic populin.

IDENTIFICATION OF SALIREPOSIDE

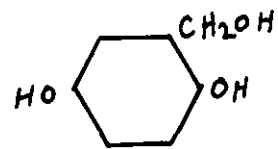
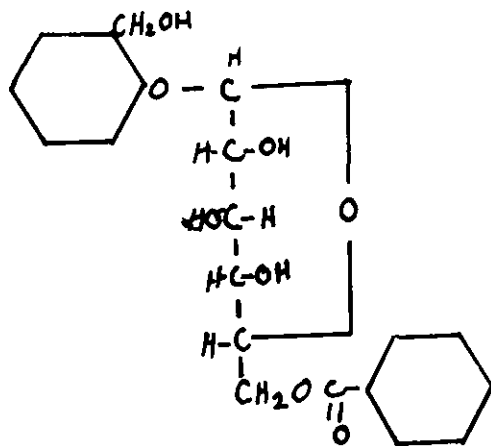
The product described on page 29 of Report No. 1 was recrystallized first from water and then from methanol to give colorless crystals shrinking at 154-156° and melting at 205°. The specific rotation was determined in 80% acetone and found to be $[\alpha]_D^{25} -35.6^\circ$ ($c = 5$). Rabaté (1) recorded a rotation of -35.5° for salireposide.

The purified compound (0.535 g.) was dissolved in a mixture of 4 ml. pyridine and 3 ml. of acetic anhydride and allowed to stand overnight. The mixture was poured into 25 ml. of cold water and stirred. The crystalline precipitate was filtered, washed with water, and recrystallized from 95% ethanol to give fine colorless needles melting at 124-126°. A mixed melting point with authentic populin acetate was depressed. Wattiez (2) reported 126.5° as the melting point of

salireposide penta-acetate. The specific rotation was determined in chloroform and found to be $[\alpha]_D^{25} -9.65^\circ$ ($c = 5$). The rotation of salireposide penta-acetate has not been recorded previously.

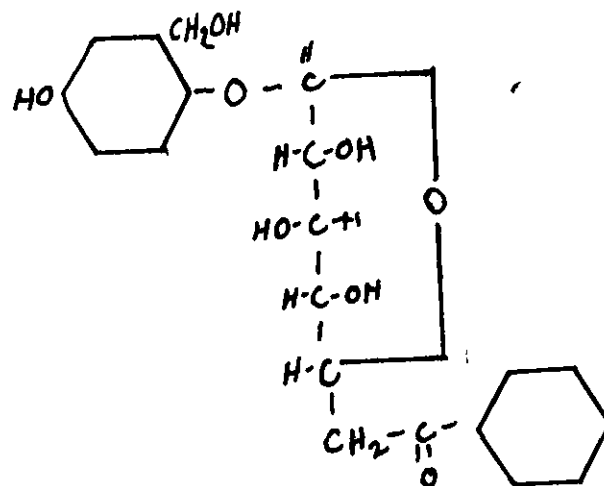
The shrinking of salireposide at 156-157° and final melting at 205° was noted by Rabaté (1) and by Sakai, Tsurumi, Eno, and Inukai (3). This melting phenomenon together with the melting point data of the acetates and the rotation data of the pure compounds indicated that our unknown glucoside was in fact identical with salireposide. The infrared absorption spectrum reported in Figure 2 of Report No. 2 was found to be identical with that of authentic salireposide obtained from Yasushi Hishiki of the Institute of Physical and Chemical Research, Komagomi Bunkyo, Tokyo, Japan. This authentic sample was that described by Sakai and co-workers (3) of the same Institute.

Salireposide was first isolated by Wattiez (2) in 1931 from a species of European willow, Salix repens, from whence the name. At that time Wattiez showed salireposide to be the benzoate of a phenolic glucoside. Later, Rabaté (1) isolated the same glucoside from Salix repens var. dumensis and hydrolyzed the product to obtain the aglucone. From its empirical formula, Rabaté inferred that this aglucone could be gentisyl alcohol. In 1943 Sakai, Tsurumi, Eno, and Inukai (3) isolated salireposide from a Japanese willow, Salix purpurea subsp. angustifolia. Later, Fujikawa and Tokuoka (4) obtained salireposide from another Japanese willow, Salix koriyanagi, proved the aglucone to be gentisyl alcohol and the glucoside linkage to involve the 2-hydroxyl group of the gentisyl alcohol. On the basis of these results, these authors assumed the structure of salireposide to be hydroxypopulin. Structures are given in Figure 1.



Gentisyl Alcohol

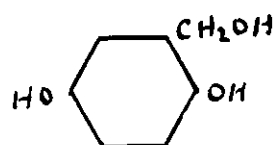
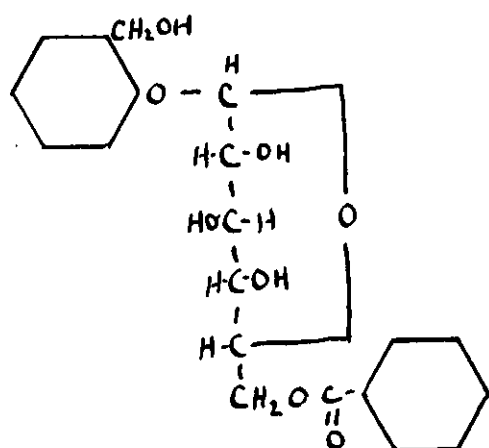
Populin



Salireposide

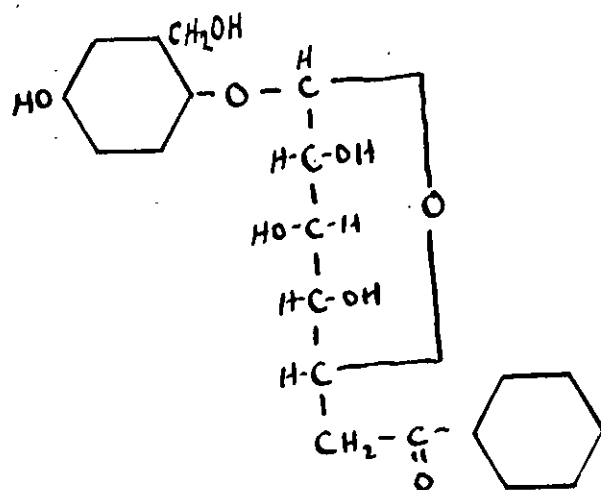
(According to Fujikawa and Tokuoka)

Figure 1



Gentisyl Alcohol

Populin



Salireposide

(According to Fujikawa and Tokunaka)

Figure 1.

REINVESTIGATION OF OTHER SUSPECTED POPULIN FRACTIONS

A number of fractions isolated from Populus tremuloides bark extracts and suspected of being populin were checked by means of paper chromatography and the DpNA spray. In all cases, no populin was present, and the compound giving the correct R_f values gave the purple color with DpNA characteristic of salireposide. In addition, a number of supposed populin acetates melting at approximately 120° were examined. Fractions N-1, M-1, and O from Figure 5 of Report No. 1 were each mixed with authentic salireposide acetate and gave no depression on melting. On the other hand, all depressed mixed melting points with authentic populin acetate. Specific rotations of these samples corresponded with that of salireposide acetate. Therefore, it appears that populin has not been found in Populus tremuloides bark.

This conclusion led to our questioning the truth of other reports of the occurrence of populin in the bark of Populus tremuloides. A search of the literature concerning such reports revealed the following. Wehmer, in his "Die Pflanzenstoffe" (5) on page 205, states salicin occurs in the bark and leaves of Populus tremuloides and populin occurs only in the bark. Wehmer's authority for these data are Braconnot (6) and Herberger (7). The Braconnot and Herberger references were studied carefully, and it was found that neither reference noted the finding of populin in Populus tremuloides, but rather of finding populin in Populus tremula, the common European quaking aspen. It must be concluded, therefore, that this statement in Wehmer is in error due to confusing Populus tremula with P. tremuloides. This error was not repeated in Klein's "Handbuch der Pflanzenanalyse" (8) on page 817 where it is reported that populin occurs in Populus tremula, P. nigra, P. pyramidalis, and P. monilifera, but it is repeated

on page 409 (9) where Wehmer and Haddes again mention populin as occurring in the bark of Populus tremuloides. Hossfeld and Kaufer (10) recently have noted the occurrence of populin in the bark extract of Populus tremuloides, but have based their observation only on the finding of benzoic acid and salicylic acid in the extracts and on analogy with Braconnot's (6) findings. That populin is present in Populus alba, Populus tremula, and in other European species seems justified by several reports in the literature and by its isolation from P. alba and P. tremula in our own laboratories in the past (see Report No. 1). However, it must be concluded from our studies and a review of the literature that populin is not present in Populus tremuloides. Experiments to date indicate that the chief glucoside components of Populus tremuloides bark are salicin, tremuloidin, and salireposide. This is the first reported instance of the presence of salireposide in any genus other than Salix.

RE-EVALUATION OF EARLIER FRACTIONS

Along with a reinvestigation of suspected populin fractions, all crystalline fractions reported in Reports 1 and 2 were submitted to paper chromatography in the BPW and EAW systems and spraying with both DpNA and Ag reagents. Many of these samples proved to be salireposide and mixtures of salireposide and tremuloidin. Many of these fractions, upon reinvestigation of melting points, demonstrated preliminary shrinking in the vicinity of 150° not recorded previously. Therefore, it became of interest to separate tremuloidin and salireposide.

Attempts at separation of tremuloidin and salireposide by fractional crystallization from water were unsuccessful. All fractions obtained were found to be mixtures by paper chromatography. Because EAW was a satisfactory

solvent for the separation of these two glucosides by paper chromatography, an attempt was made to effect the same separation on a preparative scale by means of cellulose column chromatography with the same solvent system. A cellulose column 22 mm. in diameter and 36 inches in length was prepared by slurring Whatman Cellulose powder in acetone and filling the column in small portions, allowing to drain, and tamping each portion before adding the next portion. The acetone was next displaced with EAW developer solution. A sample of mixed tremuloidin and salireposide (1 gram) was dissolved in acetone and absorbed on a little cellulose powder, and the acetone was allowed to evaporate. The sample-containing cellulose powder was placed on top of the prepared column, and the column was developed with EAW. Samples were collected in the effluent. A total of 27 samples of 15 ml. each was collected. These were spotted on paper and chromatographed in the same developer. No separation whatsoever was observed.

A search for solvent systems which would give much better separation of these two glucosides was made by paper chromatography. Systems tried included butanol saturated with water, water saturated with butanol, 1% formic acid in water, and 5% pyridine in water. The formic acid solution effected the best separation, but the results did not warrant column studies. No further studies were made at this time. Other studies on the separation of these materials on the Craig Counter Current Distribution Machine will be reported in another report.

CHEMICAL STUDIES ON SALIREPOSIDE

With substantial amounts of salireposide on hand, it became of interest to determine the complete structure of this compound. The Japanese

investigators noted above (4) demonstrated that salireposide is a benzoate of 8-gentisyl alcohol glucoside and assumed that it is hydroxy-populin with the benzoate group attached to the 6-position on the glucose (see Figure 1). At the time of this Japanese work the only known benzoate of salicin was populin, the 6-benzoate. Since then, however, we have demonstrated that the benzoate of salicin found in Populus tremuloides was not populin, but tremuloidin, the 2-benzoate of salicin (11). Thus, it occurred to us that salireposide need not necessarily be the 6-benzoate of the 8-glucoside of gentisyl alcohol, but might be the 2-benzoate, or in fact even the 3- or 4-benzoate. To locate the position of the benzoyl group, it was proposed to completely methylate salireposide, debenzoylate the methylated salireposide, hydrolyze the debenzoylated product, and identify the methylated glucose, a procedure which worked so well in the case of populin (12) and tremuloidin (11) in establishing that these two glucosides were 6-benzoyl salicin and 2-benzoyl salicin respectively.

ATTEMPTED METHYLATION OF SALIREPOSIDE--In the first experiment a mixture of 1.0 gram salireposide, 10 ml. of methyl iodide, and 8 ml. of anhydrous methanol was placed in a small flask fitted with a reflux condenser and a silicone-sealed stirrer. With stirring and boiling under reflux, 6.0 grams of freshly prepared silver oxide was added over a period of three hours. The reaction mixture was filtered, and the precipitate was washed with acetone. The yellow solution was evaporated to dryness on a rotating evaporator. The residue was covered with 10 ml. of methyl iodide, but it did not dissolve. Methanol was added until solution was complete, and the mixture was remethylated by addition of 6.0 grams of silver oxide as before. The process was repeated a third time, and the solution obtained was evaporated to yield a yellow resinous

residue, unlike a normal methylation mixture. The resinous residue was dissolved in 20 ml. of methanol and treated with a solution of 0.1 gram of sodium in 10 ml. of methanol. The mixture was boiled under reflux for 10 minutes, diluted with 30 ml. of water, and concentrated under vacuum to remove the methanol. Some crystalline material separated and was filtered. The alkaline aqueous solution was extracted with ether, and the ether was dried over sodium sulfate and evaporated to yield a colorless oil which crystallized somewhat upon standing.

The alkaline aqueous solution was acidified with dilute sulfuric acid to yield rosettes of crystals contaminated with an oily brown resinous material. The aqueous phase was decanted, and the residue was taken up in boiling water and filtered hot. The clear filtrate deposited white needles upon cooling. These were filtered and melted at 120-121°. A mixed melting point with authentic benzoic acid was not depressed. This confirms the findings of the earlier investigators that salireposide is a benzoate.

The oily crystals obtained by ether extraction of the alkaline solution were covered with 4 ml. of methanol and 6 ml. of 2N hydrochloric acid in a small flask and boiled under reflux on the steam bath for 2 hours. The methanol was removed under diminished pressure, and the hydrochloric acid was removed by stirring with some Amberlite IR-4B anion-exchange resin in the acetate form and filtering. The filtrate was evaporated under reduced pressure, and the dark syrup obtained was chromatographed on paper along with 3,4,6-trimethylglucose and a known mixture containing ribose, rhamnose, 4,6-dimethylglucose, 2,4,6-trimethylglucose, and 2,3,4,6-tetramethylglucose. The hydrolyzate showed a strong spot in the same R_f range as did the two known trimethylglucoses and a weak spot in the dimethylglucose range.

Thus, it appears from this first attempt to methylate salireposide with silver oxide and methyl iodide, that some methylation took place, but for the most part, the products were colored, uncrystallizable resins. Because this first attempt was performed with salireposide hydrate (the shrinking or preliminary melting of salireposide in the vicinity of 150° has been shown to be due to loss of water of hydration), it was thought that the presence of water from the hydrate might have caused these unsatisfactory results. Accordingly anhydrous salireposide was prepared by boiling a mixture of salireposide hydrate and toluene under reflux and water-separatory head. This procedure did produce anhydrous salireposide melting at 205° without first shrinking at $154-155^{\circ}$ as did the hydrated material. An attempt to methylate this anhydrous material by the same procedure described above for the hydrated material with silver oxide and methyl iodide lead to the same unsatisfactory colored resinous materials. Thus, the state of hydration of the salireposide was not responsible for the anomalous methylation results obtained.

Since both populin and tremuloidin could be methylated smoothly by the methyl iodide-silver oxide procedure, and since the principal difference between salireposide and both populin and tremuloidin was the free phenolic hydroxyl group of salireposide, it seemed probable that this free phenolic hydroxyl group was responsible for the anomalous methylation results. This fact was confirmed by dissolving a little salireposide in anhydrous methanol, treating with a little silver oxide, and boiling for a short while. A bright yellow color resulted. The same yellow color appeared when arbutin was subjected to the same procedure, but did not appear when populin or tremuloidin were treated in the same manner. Arbutin is the β -glucoside of hydroquinone and contains a free phenolic hydroxyl group. Thus, it appears that the free phenolic group of salireposide is responsible for the yellow resinous oxidation product

obtained when salireposide is boiled with methyl iodide in the presence of silver oxide. Therefore, in order to completely methylate salireposide for structure determination, the phenolic hydroxyl group must be methylated by some other means before complete methylation of the glucose moiety by methyl iodide and silver oxide can be performed. Experiments along this line will be described in Project Report No. 4.

HYDROLYSIS OF SALIREPOSIDE--Salireposide was hydrolyzed in accordance with the procedure described by Rabaté (1). A mixture of 0.5 gram of salireposide and 10 ml. of saturated barium hydroxide solution formed a clear pale yellow solution in the cold. The solution was boiled for 20 minutes, cooled to room temperature, treated with a few drops of 1% phenolphthalein indicator, and neutralized with dilute sulfuric acid. A little barium carbonate was added to insure neutrality, and the mixture was filtered. The precipitate was washed with water, and the combined filtrate and washings were extracted thoroughly with ether. The aqueous solution was evaporated to dryness in a rotating evaporator leaving a colorless crystalline residue having a bitter taste. This was taken up in 5 ml. of warm water and allowed to crystallize overnight. The fine white needles of debenzoylated salireposide were filtered and dried. They melted at 180° after preliminary softening at 81° and at 114°. Rabaté (1) recorded preliminary melting at 114-120° and final melting at 172° for debenzoylated salireposide, and Wattiez (2) recorded preliminary melting at 102° with final melting at 172-173°. The specific rotation found for our hydrated debenzoylated salireposide was $[\alpha]_D^{25} -42.8^\circ$. For the dehydrated material Rabaté (1) reported -56.84° and Wattiez (2) reported -57.29° . Based on a water content of 12.2% in accordance with Wattiez (2) the rotation of our anhydrous compound would be -48.7° which is somewhat lower than that reported by the previous investigators.

Debenzoylated salireposide was chromatographed in the BPW system and sprayed with DpNA. A bluish violet spot appeared at the R_f of salicin (0.52). The color produced by debenzoylated salireposide is much bluer than the spot produced by salireposide. The intense color produced by the diazo spray reagent is more evidence that the phenolic group present in salireposide and responsible for the color produced when salireposide is sprayed with the diazo reagent, is still present after hydrolysis by barium hydroxide. The relative positions of the spots for salireposide and its debenzoylated product are the same as for populin and its debenzoylated product, salicin.

The acetate of debenzoylated salireposide was prepared by reaction with acetic anhydride and pyridine. The reaction mixture was poured into water, and the product obtained was recrystallized from 95% ethanol to give crystals melting at 58-61°.

NOTE: A portion of this report on the identification of salireposide as the previously reported "populin" in Populus tremuloides has already been published in J. Org. Chem. 24, 1616 (1959).

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